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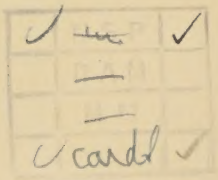

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THE FERMENTATIVE PRODUCTION OF L-VALINE BY BACTERIA*

SHIGEZO UDAKA and SHUKUO KINOSHITA

Tokyo Research Laboratory, Kyowa Fermentation Industry Company

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A very efficient procedure for production of several kinds of amino acids, such as glutamic acid, ornithine, lysine, alanine has been successfully carried out in this laboratory by means of a fermentation process (*1*). In a similar way to the other amino acid fermentations, it is now possible to produce L-valine in a high yield directly from carbohydrate and ammonia sources by the action of bacteria.

This paper describes an attempt to explain the mechanism, enabling an efficient production of valine, as well as the process of production.

METHOD AND MATERIALS

The seed medium had the following composition: 2% glucose, 1.0% peptone, 0.5% meat extract, 0.2% NaCl, pH 7.2, except that 0.2% urea was added to the medium for *Brevibacterium ammoniagenes*. Five per cent of the seed broth which was made by incubating the medium at 28°C for 24 hours with shaking was used as the inoculum to the fermentation medium. Fermentation was conducted at 28°C in a 250 ml Erlenmeyer flask in which 20 ml of the medium was dispensed. The flasks were shaken by a rotary shaker at velocity of about 180 rpm.

Unless otherwise stated, cells in a vigorous production of valine were employed for the enzymatic study. The intact cell suspension and the cell free extract were prepared as follows: Cells of one day culture for *Paracolobactrum coliforme* and two days culture for *Brevibacterium ammoniagenes* were collected by centrifugation, washed twice with 0.9% NaCl solution, and were then suspended in a 0.05 M potassium phosphate buffer, pH 7.5. The cell concentration was adjusted turbidimetrically to 50 mg dry matter per ml by measuring optical density of the suspension at 660 mμ, and then used as an intact cell suspension. The cell suspension diluted twice was treated by a 10 KC Kubota sonic oscillator for 5 to 15 minutes until a considerable disruption of cells took place. The cell debris was precipited by centrifugation at 10,000×G for 30 minutes and thus, a transparent cell free extract was usually obtained. In some cases, the ex-

* This paper was presented at the regular monthly-meeting of the Agricultural Chemical Society of Japan held on September, 1958 and June, 1959.

tract was dialyzed against 0.02 M potassium phosphate buffer, pH 7.5, using a cellophane dialyser, tubing. All extraction operations were carried out at 0~5°C.

In aerobic experiments, the reaction mixture was placed in a large test tube (diameter, 24mm) and aerated by a reciprocal shaker at 28°C.

The quantitative analysis of valine was made by the conventional bioassay technique using *Leuconostoc mesenteroides* P-60 whose growth responded exclusively L-valine. L-Glutamic acid analysis was also made by bioassay procedure using the same organism. Glucose was determined by the method of SOMOGYI (*2*). Protein was measured by a biuret method (*3*). Acetoin and diacetyl were analysed according to the method of WESTERFELD (*4*). α-Acetolactate[1] was determined as acetoin after decarboxylation by heating in a boiling water bath for 10 minutes in the presence of 1, 8 NH_2SO_4. All spectrophotometric measurements were performed with a Beckman model DU type spectrophotometer. Paper chromatographic analysis for amino acids and organic acids was usually performed by use of Tôyô filter paper No. 50 and a mixture of n-butanol 4, acetic acid 1, water 1, as the developing solvent.

L-Valine used here was the purest preparation obtained by fermentation procedure. Sodium pyruvate was prepared by neutralizing distilled pyruvic acid with sodium hydroxide and crystallization from water and ethanol. AL was prepared according to the procedure of KRAMPITS (*5*). KIV was prepared in this laboratory as follows: One mole of crude crystalline α-hydroxyisovaleric acid which was obtained from our Fuji factory was oxidized in an aqueous neutral solution with 2/3 mole of potassium permanganate at room temperature with shaking. After disappearance of the permanganate, the solution containing a bulk of MnO_2 was filtered and the clear solution thus obtained was concentrated in vacuo. The sodium salt of KIV was then prepared in crystalline form according to the method of MEISTER (*6*). α-Naphthol which was a commercial product was purified by distillation under reduced pressure in a nitrogen atmosphere. TPN and DPN were purchased from Tokyo Kasei Co. Pyridine nucleotides were reduced chemically by the hydrosulfite method (*7*). NZ-amine, an enzymatic digest of casein, was a product of the Sheffield Chemical Company.

RESULTS

Microorganisms Selected for Valine Production:

A rather large number of microorganisms that produced and accumulated valine in a large amount were obtained in the screening experiments previously described (*8*). In addition, a number of organisms isolated from natural sources were screened in the medium containing 5% glucose, 0.05% KH_2PO_4,

[1] The following abbreviations are used here: AL, α-acetolactate; KIV, α-ketoisovaleric acid; DPN and TPN, diphospho- and triphospho-pyridine nucleotide, respectively; DPNH and TPNH, reduced forms of pyridine nucleotide.

0.05% K_2HPO_4, 0.05% $MgSO_4$ 7aq, 1.0% NH_4Cl, 0.5% urea, 1.0% $CaCO_3$. As a result, the organisms which had a powerful ability to produce valine were found only in bacteria and the number of such bacteria counted more than ten.

Among these valine-producing bacteria, three strains were selected as organisms for the taxonomical study judging from their colonial appearences and productivities of valine. The strains selected were numbers 775, 749 and 40–2, and a taxonomical study was performed with reference to the *Manual of Microbiological methods by Society of American Bacteriologists*, 1957, and other references. Thus, it was revealed that strains Nos. 775, 749 and 40–2 belong to *Paracolobactrum coliforme*, *Escherichia coli* and *Aerobacter aerogenes*, respectively, according to the classification described in *Bergey's Manual of Determinative Bacteriology*, 7th Edition, 1957. The classification of other bacteria producing valine were tentatively made by gram stain, microscopic observation, colonial morphology on eosine-methylene blue medium, utilization of citrate, etc., and they include the strains of *Paracolobactrum intermedium*, *Escherichia coli* and probably the *Bacillus* species. The bacteria chosen as valine-producing strain produced 1~5 mg of valine per ml of the broth in the screening medium.

Valine-producing organisms in type culture strains are described in part in a previous paper (*8*). A gram-positive bacterium, *Brevibacterium ammoniagenes*, ATCC 6871, another valine-producing organism was selected for study of valine production.

The Course of Fermentation:

Figure 1 gives examples of the chemical changes occurring during valine fermentation. The fermentation conditions of *P. coliforme* and *Br. ammoniagenes* were chiefly studied.

P. coliforme was capable of producing valine to an extent 15% of glucose added; that is one mole of glucose was converted to 0.23 mole of valine. In fermentation of this organism, various inorganic nitrogen compounds such as ammonium sulfate, ammonia gas could be used as a nitrogen source, and urea was always effective to increase the production of valine without an apparent decomposition. The addition of certain organic nutrients in an appropriate concentration often had good influence for valine production. The nutrient not only promoted the production of valine, but also the stabilization of the fermentation. It was also found that L-leucine when applied in appropriate concentration had the same effect as organic nutrients to stabilize fermentation, and further phenylalanine, tyrosine had a similar effect. It should be noted that the amount of valine produced in some cases decreased rather sharply with a minute change of medium composition, even though no particular change was observed in cell growth, pH of the medium. *E. coli* strain 749 needed similar cultural conditions to that of *P. coliforme* for valine production. This may be understandable by the close taxonomical relationship between these strains.

The characteristics of the valine fermentation by *Br. ammoniagenes* are

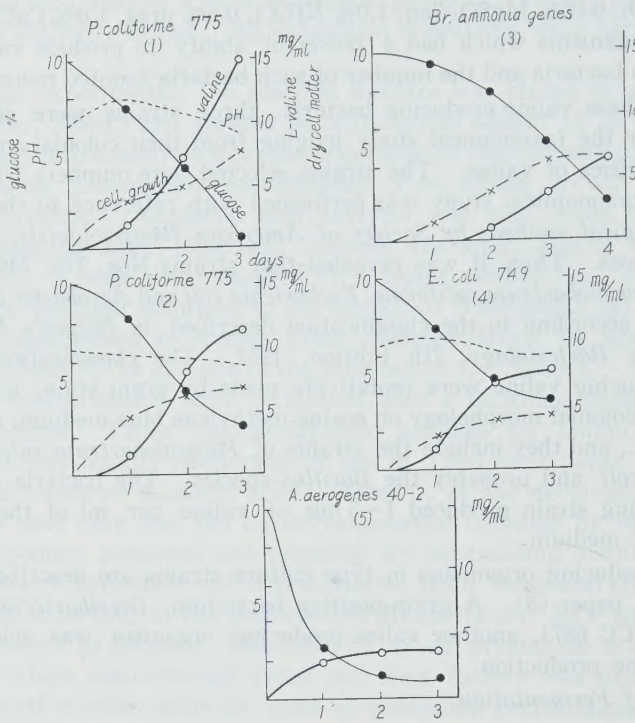

Fig. 1. Metabolic changes occurred during valine fermentation by bacteria. The composition of the media was as follows: 10% glucose, 0.1% K_2HPO_4, 0.05% KH_2PO_4, 0.05% $MgSO_4 \cdot 7aq$, 3% $CaCO_3$ were common to all media and the following components were added to each medium.

(1) 1% $(NH_4)_2HPO_4$, 1% $NH_4H_2PO_4$, 2.5% urea, 0.5% NZ-amine.
(2) 0.75% $(NH_4)_2HPO_4$, 0.75% $NH_4H_2PO_4$, 2% urea
(3) 0.5% 〃 , 0.5% 〃 , 0.2% corn steep liquor
(4) 0.75% 〃 , 0.75% 〃 , 2% urea, 0.5% NZ-amine
(5) 2.0% $(NH_4)_2SO_4$, 0.5% urea

Ammonium phosphate, ammonium sulfate, and urea were sterilized seperately and added to the medium aseptically after autoclaving. Fermentation conditions are described in the section of Materials and Methods, with the exception of a dilute urea solution added from time to time to the fermenting medium of *Br. ammoniagenes* to maintain the pH of the medium at neutrality.

that pH of the culture medium must be maintained at neutrality or slight alkalinity during fermentation and that fermentation proceeds rather slowly. An organic nutrient was essential for growth of this organism and it was assumed that some components of the nutrient are necessary for the production of valine, since the kind of organic nutrient had rather profound effect on fermentation.

In valine fermentation by these bacteria, a very small amount of amino

acids other than valine was usually found in the fermented broth. However, an increased amount of glutamic acid or alanine was often produced when valine production was inadequate, or in case cultivation was prolonged. Organic acids other than lactic acid could not usually be detected in the culture broth by a paper chromatography.

Identification of the Fermentation Product:

The amino acid in the fermented broth of *P. coliforme* strain 775 was isolated by absorption and elution on an ion exchange resin, Amberlite IR-120. After repeated recrystallizations from water and ethanol, analysis of the leaflet crystals thus obtained was made. The melting point was 310°C in a closed capillary. $[\alpha]_D^{20}$; +27.1° (C=1.0, 6N HCl). *Anal.* Calculated for $C_5H_{11}NO_2$: C, 51.26; H, 9.46; N, 11.96. Found: C, 50.8; H, 9.09; N, 12.0. These data and Rf value of the sample on paper chromatogram are in accordance with values of L-valine reported in the literature.

Although the identification of the product of other strains has not been carried out, that the product was valine was fairly strongly confirmed by the fact that the bioassay has been applied for the analysis of the product which is known to be specific to L-valine.

The Biosynthetic Pathways of Valine in P. coliforme and Br. ammoniagenes.

P. coliforme strain 775 and *Br. ammoniagenes* ATCC 6871, a gram-negative and -positive bacterium, respectively were chosen among the valine-producing bacteria for the study of valine biosynthesis.

(a) Experiments with intact Cell suspension.

The production of valine from various substrates by intact cells was investigated. The amount of valine produced by *P. coliforme* is shown in Table 1. In the same system as that of Table 1, no valine was formed from ammonium chloride and a metabolite, such as gluconate, pyruvate, lactate, acetate, citrate, fumarate, glutamate, etc. Only a minute amount of valine was produced from glucose plus ammonium chloride by some cell preparations. However, when KIV was added to the reaction mixture, valine was always produced efficiently as shown in the table. Although data in the table give a rather complicated picture, it is suggested that KIV is a precursor of valine and also that valine is not formed by the reductive amination of KIV because the amount of valine is not significantly increased by the addition of glucose or pyruvate. In the stationary incubation, aspartate has a striking effect for valine production, but this could not be explained by valine-aspartate transaminase activity as will be described later and by knowledge available up to the present. Table 1 also indicates that AL seems to be a valine precursor. In Table 2, the formation of valine by *Br. ammoniagenes* is presented. A Considerable amount of valine was formed by this organism from ammonium chloride plus glucose, lactate, pyruvate, or KIV. The addition of glucose to the reaction mixture containing KIV plus ammonium chlorides significantly increase the formation of valine. This however, could not be taken as a favorable evidence for the reductive amination of KIV, since valine was also formed from glucose plus ammonium chloride.

Table 1. Formation of valine by intact cells of *P. coliforme*.
The reaction mixtures contained 50 mg (as dry matter) of intact cells, 500 μ moles of potassium phosphate buffer, pH 6.0, 10 μmoles of $MgCl_2$, and substrates as indicated (acids were sodium salts) in a total volume of 5 ml. They were incubated at 28°C for the indicated periods.

Substrate (μ moles)	Valine formed (μ moles)					
	aerobic incubation			stationary incubation		
	1	3	8 hrs	1	3	8 hrs
None	—	0	<1	—	0	<1
KIV 100	2.1	10.7	17.1	—	—	8.6
KIV 50+NH_4Cl 200	—	32.0	33.3	—	—	—
〃 100+ 〃 〃	17.9	32.0	51.2	—	12.8	28.6
〃 〃 + 〃 〃 +Glucose 100	20.5	35.0	49.0	10.7	11.3	13.2
〃 〃 + 〃 〃 +Pyruvate 100	19.6	38.4	55.5	—	—	37.6
〃 〃 +L-Glutamate 100	12.0	29.8	62.0	—	—	14.1
〃 〃 +L-Aspartate 100	26.9	53.3	67.4	10.7	25.6	76.8
〃 〃 +D, L-Alanine 200	17.1	58.3	73.3	—	—	14.9
〃 〃 +NH_4Cl 100+Fumarate 100	—	—	—	—	24.4	35.5
AL 20	—	—	—	—	3.0	5.5
AL 20+NH_4Cl 200	—	—	—	—	2.9	5.7
〃 〃 +L-Aspartate 100	—	—	—	—	4.6	7.3

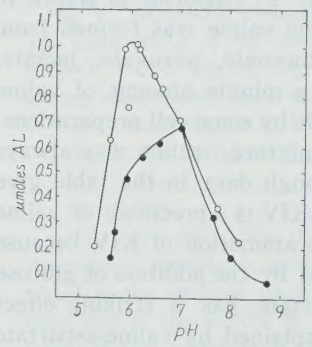

Fig. 2. PH curve of AL-forming enzyme.
The conditions were the same as the complete system shown in Table 3, except that pH of the buffer was varied. o: *P. coliforme* (0.25 mg protein) O: *Br. ammoniagenes* (0.39 mg protein) The pH indicated was that of the reaction mixture.

The enzymatic activities of each reaction step in the known pathway of valine biosynthesis were successively investigated by experiments with the cell-free extract.

(b) Formation of AL from pyruvate.

An active AL-forming system was demonstrated by either extract of *P. coliforme* or *Br. ammoniagenes*. As shown in Table 3, the reaction requires thiamine pyrophosphate as an essential cofactor. The formation of AL increased linearly with the concentration of extract, when the amount of AL formed was small (approximately less than 1.0 μ mole). Figure 2 shows the pH optimum of the reaction which is 6.0 for *P. coliforme* and 7.0 for *Br. ammoniagenes*. The activity in the extracts of both organisms was stable at −15°C at least for a few days, but only *P. coliforme* extract maintained full activity at 3°C for one day. The identity of the reaction product

Table 2. Formation of valine by intact cells of *Br. ammoniagenes*.
The reaction mixture contained 50 mg (as dry matter) of intact cells, 500 μ moles of potassium phosphate buffer, pH 7.5, 10 μ moles of $MgCl_2$, and substrates as indicated (acids were sodium salts) in a total volume of 5 ml. They were incubated at 37°C for the indicated periods.

Substrate (μ moles)	Valine formed (μ moles)			
	aerobic incubation			stationary incubation
	1	3	8 hrs	8 hrs
None	0	0	0	0
Glucose 100 +NH_4Cl 200	15.4	21.3	23.0	—
Lactate 200 + 〃 〃	15.4	23.5	27.3	—
Pyruvate 200+ 〃 〃	14.5	26.0	24.4	—
KIV 100	8.2	14.4	39.1	20.1
KIV 100+NH_4Cl 200	11.7	21.7	51.9	36.3
〃 200+ 〃 〃	14.8	25.6	65.1	40.6
〃 〃 + 〃 〃 +Glucose 100	14.4	32.9	75.9	49.2
〃 〃 + 〃 〃 +Pyruvate 100	12.5	25.6	55.4	38.5
〃 〃 +L-Glutamate 100	—	—	39.1	23.5
〃 〃 +L-Aspartate 100	—	—	40.4	23.1
〃 〃 +L-Alanine 100	—	—	34.9	23.1

Table 3. Formation of AL from pyruvate
The complete system contained 100 μ moles of potassium phosphate buffer, pH 6.0 for *P. coliforme* or pH 7.0 for *Br. ammoniagenes*, 50 μmoles of sodium pyruvate, 1 μ moles of $MgCl_2$, 1 mg of thiamine pyrophosphate, and 0.1 ml (0.12 mg protein) of the dialyzed extract of *P. coliforme* or 0.1 ml (0.39 mg protein) of the crude extract of *Br. ammoniagenes* in a total volume of 1.0 ml. The reaction mixtures were incubated in air at 37°C for one hour.

Composition	AL plus acetoin	
	P. coliforme	*Br. ammoniagenes*
complete	0.28 μmoles	0.65 μmoles
-Pyruvate	0.05	0.04
-$MgCl_2$	0.26	0.64
-Thiamine pyrophosphate	0.04	0.27
Thiamine pyrophosphate 10 γ	0.17	0.38
Double concentration of extract	0.52	1.37
Incubation period: 30 minutes	0.13	0.26

as AL is assumed by the fact that the product is decarboxylated to acetoin chemically or by the extract of *A. aerogenes* cells as described in the later section.

Table 4. Formation of valine from AL.
The reaction mixtures contained 100 μmoles of potassium phosphate buffer, pH 7.0, 5 μmoles of $MgCl_2$, 20 μmoles of substrate (acids were sodium salts), 0.5 mg of DPN, 0.1 mg of TPN were added, and 0.2 ml (protein content: 3.3 mg for *P. coliforme* and 1.4 mg for *Br. ammoniagenes*) of crude extract in a total volume of 1.0 ml. They were incubated in air at 37°C for 6 hours.

Substrate	Valine formed	
	P. coliforme	*Br. ammoniagenes*
None	<0.5 μmoles	<0.1 μmoles
AL (racemic compound)	1.7	0.1
AL+L-Leucine	1.8	0.3
〃 + 〃 +L-Glutamate+DPN	1.9	0.5
〃 + 〃 + 〃 +TPN	2.1	0.5
〃 + 〃 +D, L-Isocitrate+TPN	1.8	0.3
Ethanol+Acetate+L-Leucine	<0.5	<0.1

(c) Formation of valine from AL.

Some experimental results as shown in Table 4 suggest that AL is a probable precursor of valine. Since the amount of valine formed from AL was small and the increase of valine by the addition of AL was often much smaller than that of Table 4, no convinced evidence for the conversion of AL to valine could be obtained from such kind of experiment. As AL is well known as the precursor of valine in various microorganisms, this problem was not elaborated further.

(d) Formation of Valine from KIV.

Table 5 indicates the possibility that one of the following amino acids glutamate, leucine, or isoleucine is able to act as an active amino donor for the amination of KIV in the cell. Transaminase activities between KIV and major protein amino acids other than that in the Table were tentatively determined by the detection of valine on paper chromatograms of the reaction mixtures. None of the amino acids tested was found to be effective for the amination of KIV like glutamate or leucine. It is of interest to note that valine-leucine or valine-isoleucine transaminase activity is stronger than valine-glutamate transaminase activity in *Br. ammoniagenes*, and glutamate seems to be impermeable to the intact cell of *P. coliforme* at pH 6.0, since no apparent KIV-glutamate transaminase activity was observed by the intact cells at a lower pH.

The reductive amination of KIV was considered to be a very probable mechanism for the formation of valine from KIV. In order to demonstrate this mechanism, several probable reactions were tested in the extracts of both bacteria. As the result, no evidence for the reductive amination of KIV was obtained. The reactions tested were as follows: The crude extract unless otherwise indicated was used as the enzyme solution. (1) The reduc-

Table 5. Transaminase activities in crude extract.
The reaction mixtures contained 100 μmoles of potassium phosphate buffer, pH as indicated, 30 μmoles of substrate (acids were sodium salts), and 0.2 ml (2.1 mg protein of *P. coliforme* and 1.6 mg protein of *Br. ammoniagenes*) in a total volume of 1.0 ml. They were incubated in air at 37°C for the indicated periods.

Substrate	*P. coliforme* (at pH 6.0)			*Br. ammoniagenes* (at pH 7.5)	
	1	4	8 hrs	1	4 hrs
	valine glutamate	valine	valine glutamate	valine	valine glutamate
KIV	μmoles <1	μmoles 1.1	μmoles 0.8	μmoles <0.2	μmoles 0.3
KIV+L-Glutamate, pH 6.0	5.0	12.8	13.7	—	0.8
〃 + 〃 pH 7.5	8.0	—	13.0	0.8	1.7
〃 +L-Aspartate	—	—	2.3	—	0.5
〃 +L-Alanine	—	—	2.4	—	0.4
〃 +L-Leucine	8.1	14.3	—	—	8.9
〃 +L-Isoleucine	11.5	16.2	—	—	9.4
L-Valine+α-Ketoglutarate	8.2		12.2		1.1

tion of TPN or DPN could not be observed in the presence of valine at varied pH. (2) The oxidation of TPNH or acceleration of the oxidation of DPNH was not seen by the addition of KIV plus ammonium ion. (3) The addition of glutamate, isocitrate, fumarate, DPN, TPN, etc. (alone or in combination) to the reaction mixture containing KIV plus ammonium chloride did not cause an increase in the valine formation other than an increase in the amount of valine which was accounted by transamination. (4) In the experiment with the intact cells, the time required for the reduction of dye such as methylene blue, 2, 6-dichlorophenol-indophenol was not shortened by the addition of valine. (5) As described in section a, no favorable data for the reductive amination of KIV were obtained in the reactions with the intact cell suspension. It is, therefore, assumed that the transamination is very likely the mechanism for the amination of KIV to valine. However, there still exists the possibility that some unknown mechanisms participate in the amination of KIV.

It may thus be concluded from the experimental results described above that the biosynthetic pathways of valine in valine-producing organisms are similar to the known pathway in other microorganisms (*9*~*13*).

The Mechanism of the Production and the Accumulation of Valine.

The question raised now is how certain strains of bacteria are able to produce and accumulate a very large amount of valine in the culture medium. Some experiments were carried out in order to clarify this problem. The

Table 6. Decarboxylation of AL

The AL-forming system was the same as that given in Table 3. A portion of 0.1 ml (0.35 mg protein) of the crude extract of *A. aerogenes* ATCC 8308 was used as a source of AL decarboxylase. This extract alone did not form AL or acetoin by the system employed here for AL-formation.

Conditions	μmoles of acetoin	
	without acid treatment	with acid treatment
P. coliforme extract	0.04	0.23
P. coliforme+*A. aerogenes* ATCC 8308 extract (added initially)	0.16	0.17
P. coliforme+reaction stopped 10 minutes after adding *A. aerogenes* extract	0.27	0.26
Br. ammoniagenes extract	0.04	0.32
AL (synthetic preparation)	0	0.62
AL+*A. aerogenes* extract	0.47	0.69

following observations would provide several evidences to elucidate the efficient production of valine.

(a) AL decarboxylase activity

A. aerogenes cell is known to have a strong AL decarboxylase activity by which AL is decarboxylated to form acetoin. Since the decarboxylation of AL as it is formed would be quite unfavorable for valine formation, the AL decarboxylase activity in valine-producing strains was tested. The result are shown in Table 6 and demonstrate that valine-producing bacteria have no AL decarboxylase and that AL produced by them exclusively has one of the possible configurations.

(b) Comparison of enzyme activities in various bacterial preparations. It was assumed that valine productivity could be correlated to the increase or the decrease of enzyme activities. Data supporting this assumption are indicated in Table 7. In *P. coliforme*, the activity of the AL-forming enzyme (this will hereafter be called the condensation enzyme) varied rather sharply with even a minute change of fermentation condition and a parallelism could be seen between the amount of valine produced and this enzymatic activity. But, this was not the case for the transaminase activity. On the contrary, both the condensation enzyme and the transaminase activities of *Brevibacterium* extract did not markedly vary with the variation of growth conditions. It should be noted here that the low activity of the condensation enzyme in some of the extracts could not be ascribed to some kind of the inhibitory substance present in the extract and it probably indicates that the amount of the enzyme itself should be small, because the activity of the enzyme was not lowered by the addition of the extract with low activity.

Besides these strains, the specific activities of enzymes in other valine-producing and non-producing bacteria were measured and are also listed in

Table 7. Comparison of enzyme activities in various cultural conditions.

* The specific activities of the enzymes are indicated as follows: condensation enzyme (AL-forming enzyme); μmoles of AL/mg of protein/hour. transaminase; μmoles of L-valine/mg of protein/hour.

The activity of condensation enzyme was tested as described in the legend of Table 3, except when the pH in the reaction mixture is shown in parenthesis in case buffer other than pH of 6.0 was used. The transaminase activity was measured by the assay system given in Table 5, in which the substrates were KIV and glutamate and the pH of the buffer was 7.5.

** The composition of the basal medium is 10% glucose, 0.1% K_2HPO_4, 0.05% KH_2PO_4, 0.05% $MgSO_4 \cdot 7aq$, 3% $CaCO_3$, 0.75% $NH_4H_2PO_4$, 0.75% $(NH_4)_2HPO_4$, 2% urea (ammonium phosphate and urea were sterilized seperately). The fermentation medium of each organism is the same as described in Fig. 1. The fermentation medium of each organism is the same as described in Fig.1. The seed medium is described in the text.

Strain	Culture medium**	Culture age	Valine produced	Specific activity*	
				Condensation enzyme	Transaminase
		days	mg/ml		
P. coliforme 775	Basal medium	2	0.53	0.10	5.4
〃	〃 +0.05% L-Leucine	〃	5.9	1.8	7.6
〃	〃 +0.2% NZ-amine	〃	0	0.24	2.5
〃	〃 +0.3% NZ-amine	〃	0	0.32	2.5
〃	〃 +0.5% NZ-amine	1	3.1	3.3	8.0
〃	〃 + 〃	2	7.0	2.6	8.5
〃	〃 + 〃	3	12.0	1.3	10.1
〃	〃 + 〃 urea ommitted	2	1.4	0.18	4.1
〃	Mineral salts-glucose medium ( )	1	—	0.028 0.056 (pH 8)	6.7
〃	Seed medium	1	—	0.04	0.9
Br. ammoniagenes	Fermentation medium	3	4.7	1.4 (pH 7)	0.51
〃	Seed medium	1	—	0.53 (pH 7)	0.55
A. aerogenes 40–2	Fermentation medium	2	2.0	14.7	4.7
〃	Seed medium	1	—	9.8	1.8
E. coli 749	Basal medium	2	0.35	0.07	1.3
〃	Fermentation medium	1	1.5	1.5	4.2
〃	Seed medium	1	—	0.04	<0.3
A. aerogenes ATCC 8308	Seed medium	1	—	0.02	<0.7
E. coli K–12	Mineral salts-glucose medium	1	—	0.60 (pH 8)	2.5
〃	Seed medium	1	—	0.10	0.3
E. coli str. 15	Seed medium	1	—	0.14	1.5

Table 7. The extract of *A. aerogenes* strain 40–2 which had some activity of AL decarboxylase showed a somewhat characteristic nature in condensation enzyme; namely, such active extract exhibited activity to increase exponentially with extract concentration and showed an optimum pH at about 6.0. Since this phenomenon, as far as tested, could not be comprehended by a simple assumption, activity of the extract was estimated by the slope drawn in the region of the curve that appears to be almost linear. It is apparent that *E. coli* strain 749 belongs to the *Paracolobactrum* type and *A. aerogenes* strain 40–2 to the *Brevibacterium* type in respect to the fluctuation of the enzyme activity, and non-valine-producing bacteria have a low activity in the condensation enzyme.

Thus, the results described above may be interpreted as to indicate that a significant difference in enzyme activities between valine-producing cells and non-producing cells lies in the activity of the condensation enzyme. Since the bacterial cells in the usually have an active transaminase, and do always have the active condensation enzyme, the reaction catalized by the latter enzyme would be rate-limiting in the valine formation from pyruvate, if the assumption is made that other enzymes concerned are sufficiently active to form valine. Therefore, it follows that a high activity in the condensation enzyme in the cells would be an important factor to make the efficient production of valine possible.

It is known that the formation and the action of the condensation enzyme is inhibited by the end product, valine, in *E. coli* (*13*). This is one of the examples of the biosynthesis controlled by a negative feedback mechanism which have been well recognized in recent years. The effect of valine on the formation of the condensation enzyme in the valine-producing bacteria was apparently so complicated by the fact that the cultural conditions had a considerable influence on the formation of the enzyme. On the other hand, it became clear that the activities of the condensation enzymes of all valine-producing bacteria listed in Table 7 are not entirely influenced by L-valine of a 2×10^{-2}M concentration, but about 20~30% inhibitation was observed by 10^{-1}M valine. The inhibitation caused by such a high concentration of valine may hardly be attributed to the specific action of valine controlling the biosynthesis. This though in part, at least explains the reason why a large amount of valine produced in the fermentation medium does not suppress its own synthesis.

Whether valine once formed would be decomposed or not is another problem for its own accumulation in the medium. Since the immediate decomposition of valine did not occur at any considerable extent after the maximal production was reached, it was considered the valine-producing bacteria have a favorable nature for the accumulation of valine.

DISCUSSION

This paper is probably the first of its kind which to a certain extent reports the fermentative production of L-valine. However, a very small amount of valine has been detected in culture media of various microorganisms by several investigators who mainly studied the extracellular nitrogen compound of the organism (*14*~*16*). This phenomenon is in agreement with the view that valine is one of the amino acids which can be rather easily produced by microorganisms. In the first paper of the studies on amino acid fermentation (*8*), the authors among other facts described that certain bacteria such as *Serratia marcescens*, *Salmonella enteritidis* could produce valine in the culture medium. Very recently, SUGIZAKI also reported on the efficient production of valine by *Aerobacter aerogenes* or *A. cloacae* (*17*), and IIZUKA and KOMAGATA commented that some bacteria belonging to the family of *Enterobacteriaceae*, especially *Aerobacter*, are found to be the excellent organisms for valine production (*18*). Not only the valine-poducing bacteria listed above, but also the bacteria described in this paper mostly belongs to the family *Enterobacteriaceae*. Under such circumstances, it might be appropriate to assume that the ability to produce valine is a characteristic nature of the bacteria of *Enterobacteriaceae*.

The pathway of valine biosynthesis in microorganisms has been rather well established by the studies applying mutant, isotopic and enzymatic techniques (*9*~*13*). So far, no particular difference between the known pathways has been reported. At first, it was rather expected that some difference in respect to the pathway between valine-producing and non-producing bacteria could be found. However, the observations reported here support the view that valine in the fermentation process is formed by the biosynthetic pathway similar to that of other organisms. The appearance of glutamic acid in the broth at a laterstage of fermentation may suggest that the formation of KIV is rate-limiting in valine production, but the amino donor substance for KIV is not a rate-limiting factor.

From the experimental results found in this study it is convinced that the most probable mechanism for the efficient production of valine is reliable to the reaction of AL-forming enzyme (the condensation enzyme reported in this paper). Until recently the condensation enzyme has rather long been known to exist in the acetoin-forming system, a carboligase action (*19*), however, the enzyme is now characterized to a certain extent by demonstration of the identity of pyruvic oxidase or α-carboxylase to acyloin condensation enzyme in some cases (*20*). UMBARGER and BROWN (*13*) demonstrated in *E. coli* that the condensation enzyme described by them which had a pH optimum at 8.0 or higher was different from the enzyme of JUNI and HEYM (*20*), and they ascribed a biosynthetic role to their enzyme. The enzymes described here apparently belong to the microbial enzyme of JUNI and HEYM or the classical acyloin condensation enzyme and not to that of UMBARGER and BROWN. Whether or not the condensation enzyme active at

a lower-pH has a significant role in the biosynthesis of valine for the ordinary growning organisms is another problem, but in any way it now seems apparent that the enzyme with a low pH also plays a definite role in the biosynthesis of valine, although the synthesis of valine by such an enzyme is not controlled by valine itself.

It is of interest to note that the extract of *P. coliforme* as well as *E. coli* K–12 grown on a mineral salts-glucose medium (*13*) showed the condensation enzyme activity which is suppressed by a low concentration of valine at pH 8, but the extract of the cells grown in the basal medium of Table 7 had almost no activity at Ph 8. Therefore, it may well be stated that one of the important mechanisms for valine production is due to the presence of an active condensation enzyme which is not susceptible to valine itself.

The existence of such a biosynthetic enzyme besides "orthodox" biosynthetic enzyme described by UMBARGER and BROWN may explain the data obtained by ROBERTS et al. that the external addition of valine does not suppress its internal synthesis in *E. coli* (*21*). If the organism they studied has both enzymes mentioned above, the internal synthesis would continue by the enzyme with low pH whose activity is insensitive to the external valine.

A rather violent fluctuation in the activity of the condensation enzyme in some bacteria is noteworthy, especially from the standpoint of the production of valine. This may mean that the achievement of the increase in the condensation enzyme activity of such bacteria is prerequisite for raising the yield in valine fermentation. A similar phenomenon is known in the fermentative production of citric acid in which the enzyme activities of the biosynthetic reactions varies with the variation of the cultural conditions (*22*), and also known in some other enzymes (*23*)

Although emphasis has so far been placed on the mechanism specific for the production and accumulation of valine, it may be added that the general mechanisms which may work on amino acid fermentation (*24*), such as that the metabolic activities of the organism highly favorable for the formation of amino acid, should also apply for valine fermentation. In connection with this another important role of the condensation enzyme may be pointed out that the pyruvate which is usually produced in a large amount as an intermediary metabolite would be efficiently converted by only the active condensation enzyme into a five-carbon compound which is eventually converted to valine.

SUMMARY

1. More than ten strains of bacteria which are capable of producing large amounts of valine were selected under a screening program. The isolates from natural sources we classified as *Paracolobacterum coliforme*, *P. intermedium*, *Escherichia coli*, and *Aerobacter aerogenes*.

2. The production and accumulation of L-valine in *P. coliforme* and

Brevibacterum ammoniagenes have been investigated rather in detail, showing the formation of up to 0.23 mole of valine from one mole of glucose.

3. The biosynthetic pathway of valine revealed in the valine-producing bacteria is similar to the known pathway of other microorganisms. It was made clear by experimental results that the enzymes responsible to the condensation of pyruvate in the valine-producing cells are active at pH 5.5 7.5 and different from the "orthodox" biosynthetic enzyme which is active at pH 8, as two enzymes are known in the reaction of acetolactate formation.

4. One of the mechanisms for the efficient production and accumulation of valine is ascribed to the action of an active acetolactate-forming enzyme which is present at least in the valine-producing cells and whose action is not suppressed by valine itself.

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J. Gen. Appl. Microbiol.
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DECOMPOSITION OF BLUE-GREEN ALGAE AS EFFECTED BY THE ACTION OF SOIL BACTERIA

ATSUSHI WATANABE and TISATO KIYOHARA

The Institute of Applied Microbiology, University of Tokyo

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Several years ago, one of the present authors (WATANABE) presented a method for improving the fertility of paddy fields by the use of nitrogen-fixing blue-green algae as "green manure" (*1*). The effectiveness of this method has been proved by large-scale tests carried out on the crop yield of rice plants during the period from 1951 to 1956 at eleven experimental farms in various parts of this country; in fact, the increase in the crop attained by applying this method was, on an average, as much as 20 per cent in the final steady state (*2–4*). However, in these experiments, it was also found that the effect of the algae on crop yield was variable from one field to another, even when an equally active growth of the inoculated algae was observed in the test fields. Since it is reasonably inferred that algal nitrogen is utilized by the plant as N-source after its conversion by the activity of soil bacteria into an inorganic form such as ammonia or nitrate, the fact mentioned above indicates that the nature of the bacterial flora already present in the field is an important factor in applying this method successfully in agricultural practice.

The present work was undertaken in an attempt to obtain fundamental information as to how common soil bacteria contribute to the fertilizing action of the blue-green algae.

MATERIALS AND METHOD

Algal Materials—The nitrogen-fixing blue-green alga, *Tolypothrix tenuis*, with which our previous experiments have mainly been performed, was used as the test organism. As control, three other species of nitrogen-fixing algae, *Calothrix brevissima*, *Nostoc muscorum* and *Anabaenopsis circularis*, were also employed. The culture of these algae was run at 25–30° under illumination with incandescent or fluorescent lamps of 1000 to 3000 Lux intensity. During the culture, the algal suspensions contained in oblong flat flasks (*5*) were constantly aerated with air containing 5 per cent CO_2. The composition of the culture media used was as follows:

Medium A for *T. tenuis*, *C. brevissima* and *N. muscorum*: per liter,

KNO_3, 3 g; $Na_2HPO_4 \cdot 12H_2O$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $FeSO_4$, 0.004 g; Arnon's A_5 solution, 1 ml (pH 8.0).

Medium B for *A. circularis*: per liter, $Ca(NO_3)_2$, 0.5 g; KCl, 0.125 g; $MgSO_4 \cdot 7H_2O$, 0.0255; KH_2PO_4, 0.125 g; $FeSO_4$, 0.004 g; Arnon's A_5 solution, 1 ml (pH 8.0).

After 3 weeks of culture, the cells collected by centrifugation were aseptically washed with water and suspended in M/100 phosphate buffer (pH 8.0).

Bacterial Materials—As typical representatives of soil bacteria, twelve strains—*Bacillus subtilis* No. 11, No. 19, No. 43, H, B–208–8, *B. megatherium*, *B. proteus*, *B. cereus*, *Pseudomonas schuylkilliensis*, *P. ovalis*, *Flavobacterium sewanense* and *Achromobactor cycloclastes*—from the bacterial collection in this Institute were selected for the present purpose. These organisms were grown at 30° on a pepton-beef extract-agar medium (composition, per liter: pepton, 5 g; beef extract, 5 g; NaCl, 3 g; agar, 20 g. pH adjusted to 7.4 with NaOH). Three-day-old cultures were usually used as the test materials.

Measurement of Ammonia Production—Prior to experiments, algal samples were autolized by repeated freezing and thawing. Known amounts (4×10^7 cells) of bacteria were inoculated into each test tube of 25 ml capacity containing 10 ml of algal suspension. The inoculated tubes were shaken constantly at the rate of 200 oscillation per minute with an amplitude of 2 cm at 30°. As control, experiments were also performed with fresh algal cells.

Production of ammonia was measured with aliquots of the suspension withdrawn at an appropriate time following CONWAY's micro-diffusion technique (*6*). One ml of saturated solution of potassium carbonate was added to 1 ml of the test sample placed in a CONWAY unit. The units were left at room temperature for 3 hours, by which time the ammonia was practically completely transferred to the standardized acid solution (1 ml of N/100 HCl); this was then titrated with N/50 NaOH.

The total amount of nitrogen contained in the algal cells was determined by a conventional micro-KJELDAHL method. From the values of the total nitrogen and ammonia nitrogen produced, the percentage of decomposition of algal nitrogenous compounds was calculated.

After removing the cells and soluble proteins by treatment with 10 per cent trichloro-acetic acid, the amount of free nitrogen in the medium was also estimated by measuring the increase in the quantity of ammonia nitrogen after KJELDAHL digestion.

RESULTS AND DISCUSSION

The capacity of soil bacteria for destroying blue-green algae was examined by measuring the release of ammonia when these organisms were incubated with the algae. In Table 1, the production of ammonia from autolized cells of *Tolypothrix tenuis*, which occurred when they were mixed with a strain

of *B. subtilis* (No. 43) and *Achromobactor cycloclastes*, is presented as a function of the incubation time. As may be seen in the table, about 30 per cent of the algal nitrogen was liberated as ammonia within 3 days of incubation at 30°, but in the period thereafter the rate of ammonia production was very sluggish.

The possibility that free nitrogenous compounds other than ammonia were being formed was also examined under similar conditions by using *T. tenuis* as the alga and *B. subtilis* No. 43 as the bacterium. Compared with

Table 1. Production of ammonia from *Tolypothrix tenuis* as effected by the action of soil bacteria.

4×10^6 cells of bacteria (per ml) were incubated with autolized cells of *Tolypothrix tenuis* (containing 0.050 mg of nitrogen per ml) in M/100 phosphate buffer (pH 8.0) at 30°. Quantity of ammonia produced is given as γ N per 1 ml of suspension (left column) and as per cent of total algal nitrogen initially supplied (right column). See text for other conditions of experiment.

Incubation time (days)	Ammonia produced			
	Bacillus subtilis		*Achromobactor cycloclastes*	
	γ	%	γ	%
3	14.8	29.5	12.0	24.0
6	13.0	26.0	14.0	28.0
9	20.0	40.0	15.0	30.0
12	19.5	38.4	17.0	33.6

Table 2. Comparison of activity of various soil bacteria in producing ammonia from fresh and autolized cells of *Tolypothrix tenuis*.

All figures represent ratio of ammonia nitrogen produced by action of bacteria to total algal nitrogen initially supplied (0.057 mg per ml). Incubation period: 10 days. See text and legend in Table 1 for other conditions of experiment.

Bacteria	Fresh cell	Autolized cell
Bacillus subtilis No. 11	5.5	42.7
〃 〃 No. 19	12.9	25.0
〃 〃 No. 43	9.3	26.2
〃 〃 H	14.6	19.5
〃 〃 B-208-8	7.5	19.5
Bacillus cereus	15.5	24.5
Bacillus proteus	5.5	31.5
Bacillus megatherium	13.9	25.0
Pseudomonas ovalis	31.0	22.3
Pseudomonas schuylkilliensis	11.3	20.5
Flavobacterium sewanense	16.7	22.3
Achromobactor cycloclastes	11.3	25.3

ammonia, however, only a negligibly small increase of these substances (less than 1 per cent) was observed during the incubation period.

Since practically no net formation of ammonia was observable in a period of more than 9 days, in subsequent experiments the amount of ammonia formed during 10 days of incubation was used to compare the activity of various strains of bacteria. The results obtained with *T. tenuis* are shown in Table 2. A far more marked formation of ammonia (about 2 to 8 times) was always detectable with autolized cells as compared with fresh ones. Although all bacterial strains tested were proved to be more or less active in producing ammonia from the cells of *T. tenuis*, their activity was variable from strain to strain; the most prominent activity was found in a certain strain of *Bacillus subtilis* (No. 11), by the action of which over 40 per cent of the algal nitrogen was converted to ammonia within 10 days.

Quite similar features were also observed with other species of nitrogen-fixing blue-green algae (Table 3).

Table 3. Comparison of activity of various soil bacteria in producing ammonia from autolized cells of 3 species of blue-green algae.

All figures represent ratio of ammonia nitrogen produced by action of bacteria to total nitrogen initially supplied (0.16–0.3 mg per ml). Incubation period: 10 days. See text and legend in Table 1 for other conditions of experiment.

Bacteria	*Calothrix brevissima*	*Nostoc muscorum*	*Anabaenopsis circularis*
Bacillus subtilis No. 19	4.3	20.9	15.3
〃 〃 No. 43	5.4	20.6	7.1
〃 〃 H	11.6	19.7	12.6
〃 〃 B-208-8	4.3	18.8	12.6
Bacillus cereus	5.7	15.6	12.0
Pseudomonas ovalis	3.4	18.3	12.0
Pseudomonas schuylkilliensis	6.2	21.6	14.1
Flavobacterium sewanense	6.1	14.8	14.2
Achromobactor cycloclastes	25.6	18.8	18.6

These results make it probable that the fertilizing action of nitrogen-fixing blue-green algae in the field may also be considerably influenced by the bacterial flora, at least with respect to their effectiveness as a nitrogenous nutrient. In nature, however, fertilizing effect of the algae is believed to be far more complicated than this, since the constituents of algae other than the nitrogenous ones must also be utilizable as nutrients of the plants.

A further problem which remains to be solved is how to produce a field condition favorable to the growth of the algae as well as to that of the bacteria which are presumably playing an important role in making the algal constituents available to higher plants.

SUMMARY

The decomposition of the nitrogen-fixing blue-green algae, *Tolypothrix tenuis*, *Calothrix brevissima*, *Nostoc muscorum* and *Anabaenopsis circularis*, brought about by the action of various soil bacteria was examined. A certain strain of *Bacillus subtilis* was found to give the most active production of ammonia; about 40 per cent of the algal nitrogen being converted to ammonia within 10 days of incubation. Practically no production of nitrogenous compounds other than ammonia was observed. On the basis of these results, the varying efficacy of blue-green algae as fertilizer in paddy fields was discussed.

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J. Gen. Appl. Microbiol.
Vol. 5, No. 4, 1960

STUDIES ON THE CLASSIFICATION OF STREPTOMYCETES, PART XII* THE MICROSCOPIC MORPHOLOGY AND OTHER CHARACTERISTICS OF *STREPTOMYCES HYGROSCOPICUS* AND MORPHOLOGICALLY RELATED STRAINS

RYOSAKU NOMI

Faculty of Engineering, University of Hiroshima

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Streptomyces are, as it is often pointed out, morphologically well differentiated and similar to fungi. In 1919, DRECHSLER (*1*) reported considerably detailed morphological characteristics of *Actinomyces*, but the study received few attention. Since then, WAKSMAN et al. (*2*), KRASSILNIKOV (*3*), BALDACCI et al. (*4*) and GAUSE proposed taxonomic system which attached great importance to physiological and cultural properties and which more or less made light of morphological properties. In 1958, PRIDHAM et al. (*5*) proposed the subdivision of the genus *Streptomyces* into several morphological sections. They have successfully adopted morphology of sporiferous part as a taxonomic criterion. Recently, ETTLINGER et al. (*6*) reported another taxonomic system in which morphological properties were adopted as important criteria.

Now, the author considers that, in morphology of *Streptomyces*, not only sporiferous part, but also other parts of aerial mycelia have morphological characteristics distinctly enough to be adopted as taxonomic criteria.

TRESNER and BACKUS (*7*) investigated various morphological and physiological characteristics of eighteen strains of *Streptomyces hygroscopicus* and their related species. They concluded that the most constant characteristics in *Streptomyces hygroscopicus* were spore color *en masse*, tightly wounded coils of the spore-bearing hyphae arisen as short side branches of main hyphae and the black hygroscopic areas developed on colonies. They also pointed out that other various cultural and physiological features were variable.

The author (*8*~*15*) has already reported on the various types of morphology in *Streptomyces* and has pointed out that microscopic morphology is a stable characteristics. In this paper, the author intends to report on the detailed microscopic morphological characteristics of *Streptomyces hygroscopicus* and their morphologically related strains. The morphological characteristics presented in this paper is a new morphological type in *Streptomyces* which differs from the other morphological types reported in the previous papers.

* Parts I–XI have been published in *J. Ferment. Tech.*, Japan, **31** (1953) **32** (1954) and **33** (1955).

EXPERIMENTS AND RESULTS

I. Strains employed in this study

The strains employed are *Streptomyces hygroscopicus* (JENSEN) WAKSMAN et HENRICI HUT 6026 [←IFO 3192←CBS (Westerdijk)], *Streptomyces violaceoniger* HUT 6088 [←IAM←NRRL B-1477], *Streptomyces albus* (Rossi Doria emend. Krainsky) WAKSMAN HUT 6047 [←IAM W5-9←MTHU←ATCC 618 (A. J. Kluyver)] and six other strains which were isolated from the soil at this laboratory. While these six strains are called by strain number, they may be identified with already published type cultures. But in author's collection of type cultures no same cultures as any of these six strains can be found.

Nowadays, specification of species is usually described according mainly to cultural and physiological characteristics of strain, while the morphological characteristics are not always described in detail. But the author considers it desirable to adopt more than ever morphological characteristics as taxonomic criteria. Accordingly, when the author wishes to identify some strain with an already published type strain, he must study the detailed morphological characteristics of the type strain. Because, when the identification was made using only the cultural and physiological criteria and not using detailed morphological criteria, there may be possibility to identify the isolated strain with a type strain, though they are apparently of morphological difference. Such case can be instanced in the author's collection of type cultures itself. Therefore, untill the author obtains the type strain which, both morphologically and physiologically, is considered to be of the same species with the author's unnamed strain, the author postpones the denomination of above six strains.

II. Method of microscopic observation

The detailed observation of microscopic morphology was carried out using cover culture method (*16*) together with plate observation method. The medium used is Bennett's agar or modified Bennett's agar. In Bennett's agar glucose is used as carbon source, but glucose is not always an ideal carbon source for all strains to produce the aerial mycelium. As will be made clear later in this paper, the c-source for good production of aerial mycelium varies as to strains employed. In morphological study the suitable c-source need to be used. Usually glycerol, xylose, glucose, fructose, mannitol, sucrose, maltose, lactose and starch are examined. In modified Bennett's agar c-source is favourably substituted for glucose in Bennett's agar. If necessary, the concentration of c-source is modified to desired consistency such as half or three times as much as original concentration. When the quality or quantity of c-source is inadequate, the microscopic characteristics show the incompletely grown morphology whose subsequent growth has been suspended.

III. Cultural characteristics

The slant cultural features on Czapek-Dox agar, Krainsky agar, Bennett agar and modified Bennett agar, and starch hydrolysis test and behavior on skim-milk and nitrate reduction test of each strain were studied and recorded. Incubation was carried out for fourteen days at 28–30°C, with the exception of seven days incubation in starch hydrolysis test. The data will be given at the end of this paper, together with some special morphological features of each strain, under "Description of Strain".

It is almost a tradition among mycologist that in classification of microorganism the complete morphological growth phase should be made to research first of all and with as much exertion as possible. And the color of aerial mycelium should always be referred to in connection with the morphology of sexual organ. Now, the author considers that, in *Streptomyces*, great importance is to be given to the color of aerial mycelium when the full morphology has been formed and the spores have matured. In *Streptomyces*, even if the full morphology has been formed, the color of aerial mycelium may vary in some degree when the cultural medium is changed and often even on a slant culture. However, the variation of color in those cases occurs within some certain range of a color series. Therefore, in describing the color of culture, it is desirable to express in terms of some width of color. Moreover, if any other special color occurred at some period of growth or on some cultural medium used, the color should be expressed as an instance of special reference.

IV. Microscopic morphology

Morphological characteristics of each strain employed are not just the same, having, as they do, some special features which are different from each other. It is described at the end of this paper together with the cultural characteristics. On the other hand, each strain has general morphological features which are common to all strains employed and which apparently distinguish themselves from other types of morphology in *Streptomyces*, the latter has already been reported and will be reported in succeeding papers. In this section, reference is solely made to the general morphology of the strains employed.

General morphology of the strains employed: The aerial mycelia are well developed but the branching system is not simple. The aerial mycelia sometimes take the shape of comparatively long straight or long wavy and sometimes short straight or short wavy, and they branch in monopodial alternate, opposite and verticillate forms especially in their young stage of growth. But in accordance with their growth the branching system occasionally becomes to seem irregular. It is because, when the aerial mycelia have well developed and repeatedly branched, the degree of development and the direction of branches are not uniform and sometimes the direction of branches at distal parts is just opposite to it at starting part. Furthermore, branching form of aerial mycelia sometimes seems like sympodial system.

And on the whole, there are no main axial mycelia, and repeatedly diverged branches are still generally axial mycelia. Occasionally, under low microscopic magnification, they seem cluster-like or coelicolortype (*8*)-like. Now, what is significant and noticeable is that they do not break into oidiospores. Sporiferous parts are, indeed, side branches which arise from those various axial mycelia. Sometimes long aerial mycelium earths on the medium, from which point vegetative mycelium grows on again. As the result of those growth system mentioned, no regularity is often observed in branching system of aerial mycelia after good growth, and the microscopic appearance of them occasionally appears net-like at a glance.

The monopodially or irregularly branched axial mycelia give rise to side branches which branch on further in various degree, i. e. from single to much diverged and complicated branches. The side branches develop into spirals or wavy hyphae in terminal filaments which break into oidiospores. The number of branches in a side branch is not constant in a strain and length of terminal spirals or wavy hyphae also differs from strain to strain and so it does within a strain. When a side branch is thickly branched, its morphology, in itself, is similar to the morphological type of *Streptomyces coelicolor* et al. (*8*, *9*, *13*)

In spirals, the number of winding, loose or compact, and dimension of diameter are about constant in a strain within certain range, which is different from strain to strain. The direction of turn in spiral, namely dextrorse or sinistrorse, is exclusively constant in a strain. In all strains employed in this study, it is sinistrorse. The direction of turn is decided by tracing the spiral in this direction from far to near. (In author's previous papers, (*8*～*14*) the direction of turn was decided by tracing in contrary direction to it in this paper. But, as the author has found the previous method botanically mistaken, it is amended from this paper on.) In terminal spiral parts of some strains employed, a sympodially branched chain of a few spirals is sometimes observed.

Oidiospores are of various shape. In *Streptomyces*, the shape of oidiospores is either sphere, ellipsoid, short rod or rod. The shape of oidiospores in a strain is not only of a kind of shape, but principally contains various shapes. In spiral's coiled parts or short wavy sporiferous parts, the shape of oidiospores is generally sphere to ellipsoid (or short rectangle), and in long straight sporiferous parts, it generally contains many longer shapes, i.e. from ellipsoid to short rod to rod. As the shape of oidiospores is correlative with morphology of sporiferous parts, the shape of spores is not always significant in taxonomic criterion.

In this morphological type, there is often contained some transitional types which is different from the above-mentioned morphology. On the colonial part bearing long aerial mycelia in abundance, the morphology resembles that of *Streptomyces sp.* No. 477 (*14*) reported in the previous paper. In the latter, long straight aerial mycelia project radially, branching in monopodial alternate, opposite and verticillate forms, and the branches follow

the same branching pattern. The long straight aerial mycelia do not break into oidiospores but give rise to short side branches, which branch, on further, in various degree, i.e. from single to much diverged branches. The terminal filaments of these side branches form spirals or short wavy filaments and sporulate.

On the colonial part bearing no long aerial mycelia, the morphology resembles that of *Streptomyces coelicolor* et al. (*8*, *9*, *13*) reported in the previous papers. In the latter, the aerial mycelia are comparatively straight or wavy in shape and branch in monopodial alternate, opposite and verticillate forms, and the branches follow the same branching pattern in various degree. The terminal filaments give rise to spirals or short wavy filaments and sporulate.

In the morphological type presented in this paper, those two transitional morphology and the above-mentioned irregularly branched morphology are simultaneously included in various ratio and such ratio differs from strain to strain.

Now, therefore, the occurrence of morphological segregation may be expected in this morphological type; for instance, some strain studied may morphologically segregate to *Streptomyces sp.* No. 477 type and *Streptomyces coelicolor* type. But the author's experiment has denied the segregative possibility, to which some experimental data will be given in the following paper.

The appended photographs will be made available for understanding general morphology of the strains studied.

DESCRIPTION OF STRAIN

For experimental items, see the sections of "Cultural characteristics" and "Microscopic morphology". In the following description, the abbreviation, (A), (V), (R) and (P), mean aerial mycelium, vegetative mycelium, reverse of colony and soluble pigment, respectively. On the aerial mycelium, the degree of development is expressed in terms of five grades; non, slight, moderate, fairly good and good. Italic color name is quoted from RIDGWAY's Color Standards and Nomenclature 1912. In modified Bennett's agar, favourable carbon sources are shown in round brackets. Although the color of aerial mycelium on the morphologically unfavourable cultural medium is not necessarily significant in taxonomic study, all the cases of color will be referred to when aerial mycelium is produced for the sake of comparison with other experimental data.

In the following description the name of slant cultural medium is curtailed "agar".

Streptomyces hygroscopicus HUT 6026

I. Long axial mycelia are fairly developed and either straight or a

little flexous. They are much longer than side branches. Sometimes axial mycelia are short in length.

Side branches are single to about three step' branching with 5 to 8 terminal hyphae in many cases, but occasionally more abundantly branched. When abundantly branched, it is impossible to distinguish them from short axial mycelium with short side branches. Side branches arise thickly from axial mycelium. Terminal hyphae in side branches are short in length.

Spiral: sinistrorse, very compact (each ring of spiral is adhered to compact (each ring not adhered), 1 to 6 turns, 2.5 to 5 μ in diameter, mostly cylindrical and sometimes snail-like. Length of spiral (including spiralphore) is 3 to 12.5 μ. **Spore**: sphere to ellipsoid to rectangle, 1.0 to 1.4 by 1.0 to 1.8 μ in diameter.

Characteristic moist and dark brownish hygroscopic areas develop on colonies.

II. Czapek: (A) fairly good; white. (R) brownish white. (P) almost non. **Krainsky**: (A) slight; white to a few patches of *Hair Brown*. (R) yellowish white. (P) non. **Bennett**: (A) slight; white to a few patches of brownish black. (R) pale yellow. (P) non. **Mod. Bennett** (xylose and maltose): (A) good; *Drab-Gray*, *Light Drab* and *Mouse Gray*→*Fuscous*→*Fuscous-Black*, *Seal Brown* or *Blackish Brown* (*1*) patches when self-moistened. (R) pale yellow. (P) non.

Starch: Hydrolysis positive. **Milk**: No coagulation; peptonization positive. **Nitrate**: Reduction very weak or negative.

***Streptomyces violaceoniger* HUT 6088**

I. Almost the same as *S. hygroscopicus* HUT 6026. In comparison, long axial mycelia are small in number and turns of spiral are few.

Spiral: sinistrorse, very compact to compact, 1 to 4 turns, 2.5 to 5 μ in diameter, almost cylindrical. Length of spiral is 3 to 9.5 μ.

Hygroscopic areas develop and are almost black.

II. Czapek: (A) non. (V) colorless to slightly yellow. (R) pale yellowish brown. (P) pale brownish yellow. **Krainsky**: (A) non. (V) pale yellow to yellow. (R) pale yellow to yellow. (P) almost non. **Bennett**: (A) slight: white. (R) yellowish orange. (P) almost non. **Mod. Bennett** (xylose): (A) fairly good; *Smoke Gray* to *Pale Mouse Gray*→*Pale* (or *Light*) *Purplish Gray*, *Pale Neutral Gray*→moist black patches when self-moistened. (R) yellow to yellowish brown. (P) almost non.

Starch: Hydrolysis positive. **Milk**: No coagulation; peptonization positive. **Nitrate**: Reduction negative.

***Storeptomyces albus* HUT 6047**

I. In early stage of growth, the branching system is almost similar to *S. coelicolor* et al. But in its stage of development, the non-sporulated axial filaments become much longer in length to exceed that of side branches, their shape being not straight but more or less flexous.

The terminal hyphae in side branches are longer than those of the above two strains.

Spiral: sinistrorse, very compact to compact, 1 to 5 turns, 3 to 6 μ in diameter. Length of spiral is 4 to 18 μ. **Spore**: ellipsoid to short rod, 0.6 to 0.9 by 0.7 to 1.4 μ in diameter.

II. Czapek: no growth. **Krainsky**: (A) moderate; white. (R) white to yellowish white. (P) non. **Bennett**: (A) good; white. (R) yellowish brown. (P) pale yellow. **Mod. Bennett** (all 9 c-sources):(A) good; white. (R) yellow or yellowish brown. (P) non to pale yellow.

Starch: No growth on ammonium sulfate medium. On starch-Bennet. agar and starch-Krainsky agar plate, starch is not hydrolyzed. **Milk**: No coagulation; peptonization positive; red soluble pigment. **Nitrate**: Reduction negative or very weak.

***Streptomyces sp.* No. 201**

I. About the same as *S. albus* HUT 6047.

Spiral: sinistrorse, very compact, 1 to 4 turns, 2.5 to 5 μ in diameter, Length of spiral is 6 to 20 (occasionally 30) μ. **Spore**: ellipsoid to short rod. 0.7 to 1.2 by 1.0 to 1.4 μ in diameter.

Rarely moist black areas develop on the arial mycelium.

II. Czapek: (A) almost non. (V) colorless. (R) colorless. (P) non. **Krainsky**: (A) good; brownish white to *Light Drab*. (R) white to brownish white. (P) non. **Bennett**: (A) fairly good; white to *Pale Mouse Gray* or *Drab-Gray*. (R) brownish yellow to yellowish brown. (P) non. **Mod. Bennett** (glycerol, fructose and maltose):(A) good; *Drab-Gray*, *Smoke Gray* to *Light Mouse Gray*. (R) yellowish brown. (P) pale brown.

Starch: Hydrolysis negative. **Milk**: No coagulation; peptonization positive; deep brownish black soluble pigment. **Nitrate**: Reduction positive.

***Streptomyces sp.* No. 350**

I. About the same as *Streptomyces albus* HUT 6047 and *Streptomyces sp.* No. 201, except the growth of long axial aerial mycelium is more prosperous than that of above two strains.

Spiral: sinistrorse, compact (to very compact), 1 to 5 turns, 3 to 6 μ in diameter. Length of spiral is 6 to 37 μ. **Spore**: sphere to ellipsoid, 0.8 to 1.2 by 0.8 to 1.4 μ in diameter.

II. Czapek: (A) good; white to *Drab-Gray*. (R) pale brownish yellow. (P) almost non. **Krainsky**: (A) good; *Pale Drab-Gray* to *Drab-Gray*. (R) grayish white. (P) non. **Bennett**: (A) good; white to *Pallid Mouse Gray*. (R) orangish yellow to yellowish brown. (P) non. **Mod. Bennett** (glycerol, mannit, starch and maltose): (A) good; *Pallid Mouse Gray* to *Smoke Gray* to *Drab-Gray*. (R) orangish yellow to yellowish brown. (P) non.

Starch: Hydrolysis positive. **Milk**: No coagulation; peptonization positive; no soluble pigment. **Nitrate**: Reduction negative.

***Streptomyces sp.* No. 202**

I. This strain's long axial aerial mycelium is better developed than that of *Streptomyces sp.* No. 350 and occasionally shows resemblance to *Streptomyces sp.* No. 477 as reported previously. Furthermore, a single growth unit of aerial mycelium having no long mycelium is well branched and has resemblance to *Streptomyces sp.* No. 189[13] previously reported. These two extremes in morphology are much more conspicuous than in the other strains studied. Of course, the already mentioned general morphological units are large in number. Most terminal hyphae are spiraled, while non-spiraled wavy hyphae are few.

Spiral: sinistrorse, very compact to compact, 2 to 6 turns, 3 to 6 μ in diameter, cylindrical to occasionally snail-like. Length of spiral is 6 to 22 μ. **Spore**: sphere to ellipsoid, 1.0 to 1.4 by 1.0 to 1.6 μ.

II. Czapek: (A) fairly good; white to *Light Olive-Gray*. (V) orange. (R) orange→yellowish brown. (P) vivid yellow. **Krainsky**: (A) good; gray concentrated than *Light Drab*. (R) yellow→yellowish brown. (P) vivid yellow →yellowish orange. **Bennett**: (A) good; *Smoke Gray*, *Light Grayish Olive* or *Tea Green*→*Light Drab*→*Hair Brown*, *Benzo Brown*. (R) yellow→brown. (P) vivid yellow→yellowish orange. **Mod. Bennett** (all 9 c-sources): same as Bennett.

Starch: Hydrolysis positive. **Milk**: No coagulation; peptonization positive. **Nitrate:** Reduction negative.

***Streptomyces sp.* No. 602**

I. Peculiar spirals are observed in this strain. Before sporulation, they are compact, but after sporulation they develop into various shapes, namely from long open to very compact. Furthermore, even in a spiral there is often included various shapes ranging from long open to very compact when sporulated, resulting in random spiral formation.

Spiral: sinistrorse, open to very compact (often random), 4 to 10 turns, 2.5 to 4.5 μ in diameter. Length of spiral is 6 to 40 μ. **Spore** sphere to ellipsoid, 0.6 to 1.0 by 0.8 to 1.6 μ in diameter.

II. Czapek: (A) moderate; white to gray. (V) colorless. (R) brown. (P) brown. **Krainsky**: (A) moderate; gray. (R) color of aerial mycelium is looked through. (P) non. **Bennett**: (A) moderate; *Drab-Gray* to *Light Drab*. (R) pale yellow to brown. (P) yellowish brown. **Mod. Bennett** (fructose, maltose and starch):(A) good; gray concentrated than *Light Drab* or intermediate color between *Drab* and *Hair Brown*. (R) pale yellow to dark brown. (P) almost non.

Starch: Hydrolysis positive. **Milk**: No coagulation; peptonization positive. **Nitrate**: Reduction negative.

***Streptomyces sp.* No. 469**

I. The morphological features are in conformity with general morphology.

Spiral: sinistrorse, compact (occasionally very compact), 2 to 8 turns, 3

to 4 μ in diameter. Length of spiral is 4 to 18 μ. **Spore**: ellipsoid to short rod, 0.7 to 1.0 by 0.8 to 1.6 μ in diameter.

II. Czapek: growth weak. (A) non. (V) colorless. (R) colorless. (P) non. **Krainsky**: (A) good; (*Pale* or) *Light Vinaceous-Lilac* to *Pale Vinaceous-Drab*. (R) dark reddish violet. (P) brown. **Bennett**: (A) good; white→purplish white→*Drab-Gray*→*Benzo Brown*→*Blackish Brown* (*I*). (R) dark reddish violet. (P) brown. **Mod. Bennett** (all 9 c-sources): (A) good; white→pale reddish purple→*Drab-Gray*→*Benzo Brown*, *Fuscous*, *Fuscous-Black* or *Blackish Brown* (*I*) when self-moistened. (R) dark reddish violet. (P) brown.

Starch: Hydrolysis positive. **Milk**: No coagulation; no peptonization; brownish gray soluble pigment. **Nitrate**: Reduction positive.

***Streptomyces sp.* No. 203**

I. This strain's side branches are gigantic and often difficult to distinguish them from axial parts. Spirals are also gigantic.

Spiral: sinistrorse, open to compact (sometimes open to compact parts are contained in a spiral), 2 to 6 turns, 3 to 9 μ in diameter. Length of spiral is 15 to 100 μ. **Spore**: ellipsoid to short rod to a little long rod, 0.6 to 1.0 by 1.0 to 1.6 μ in diameter.

II. Czapek: (A) moderate; white. (R) pale yellow to orange. (P) almost non. **Krainsky**: (A) moderate; white. (R) white. (P) non. **Bennett**: (A) slight to moderate; almost white. (R) pale orange. (P) pale yellow. **Mod. Bennett** (mannit, sucrose, maltose and starch): (A) good; *Shell Pink*, *Pale Vinaceous-Pink* to *Pale Grayish Vinaceous*. (R) yellow to brown. (P) yellow to yellowish brown.

Starch: Hydrolysis negative. **Milk**: No coagulation; peptonization negative or very weak; brownish gray soluble pigment. **Nitrate**: Reduction negative.

SUMMARY

The microscopic morphology of *Streptomyces hygroscopicus* and morphologically related strains are described. These strains have sinistrorse spirals in terminal filaments of side branches arisen from axial mycelia. Axial mycelia are fairly long but sometimes short, straight to flexous, and the branching system principally takes various monopodial forms in its early stage of growth, but often becomes to appear irregular after full growth has been attained. Side branches range from single to branched.

Description of each strain employed is also given.

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EXPLANATION OF PLATES I–III

Fig. 1 and Photos. 1–4 are *S. hygroscopicus* HUT 6026. Fig. 1 is the sketch. Photo. 1 (photographed with 100× microscopic magnification) shows a part of colony. Photo. 2 (photographed with 450×) shows appearance of short side branches arising from axial filaments. Photo. 3 (450×) shows appearance of a part of colony bearing thickly branched side branches. Photo. 4 (1000×) shows very compact spirals.

Photos. 5–7 are *S. violaceoniger* HUT 6088. Photo. 5 (100×) shows a part of colony. Photo. 6 (450×) shows appearance of short side branches arising from axial filaments. Photo. 7 (450×) shows a part of colony bearing thickly branched side branches.

Photos. 8–10 are *S. albus* HUT 6047. Photo. 8 (100×) shows appearance of branching axial mycelia where short side branches are not yet borne. Photo. 9 (100×) shows appearance of a part of colony where axial mycelia have already borne many side branches with spirals. Photo. 10 (450×) shows a part of colony where short side branches are attached to axial mycelia. The short side branches are longer than those of the above two strains.

Photo. 11 (450×) is *Streptomyces sp.* No. 201 showing a part of colony where short side branches are attached to flexous axial mycelia.

Photos. 12–14 are *Streptomyces sp.* No. 350. Photo. 12 (100×) shows a part of colony in which are seen young growth unit having a few side branches as well as well developed growth unit having fairly many side branches. Photo. 13 (450×) shows side branches attached to flexous axial mycelia. Photo. 14 (450×) shows thickly branched side branches attached to axial mycelia.

Photos. 15–19 are *Streptomyces sp.* No. 202. Photo. 15 (100×) shows a colonial part with a little flexous axial mycelia bearing side branches. Photo. 16 (100×) shows a colonial part with straight axial mycelia bearing side branches. Photo. 17 (100×) shows growth units which have no long axial mycelia but the morphology resembles S. coelicolor-type. Photo. 18 (450×) shows side branches attached to axial mycelia. Photo. 19 (450×) shows a coelicolor-type's growth unit.

Photos. 20–23 are *Streptomyces sp.* No. 602. Photo. 20 (100×) shows a part of colony. Photo. 21 (450×) shows side branches attached to axial mycelia. Photo. 22 (450×) shows a simple growth unit which is not a side branch, while Photo. 23 (450×) shows a well developed side branch, and in those two photographs various forms of spirals are observed.

Photos. 24–26 are *Streptomyces sp.* No. 469. Photos. 24 and 25 (both 100×) show appearance of a part of colony where various growth stages are seen which range from axial mycelia bearing no side branches to those bearing many side branches. Photo. 26 (450×) shows side branches attached to axial mycelia.

Photos. 27–30 are *Streptomyces sp.* No. 203. Photo. 27 (100×) shows a part of colony and Photo. 28 (100×) shows a growth unit. Photos. 29 and 30 (both 450×) show side branches attached to axial mycelia. Since the side branches in this strain are gigantic, it is not easy to contain side branches and axial mycelia together in one photograph.

One unit of the scale in the photographs is 11 μ in 100×'s photographs, 2.5 μ in 450×'s photographs and 1.1 μ in 1000×'s photographs.

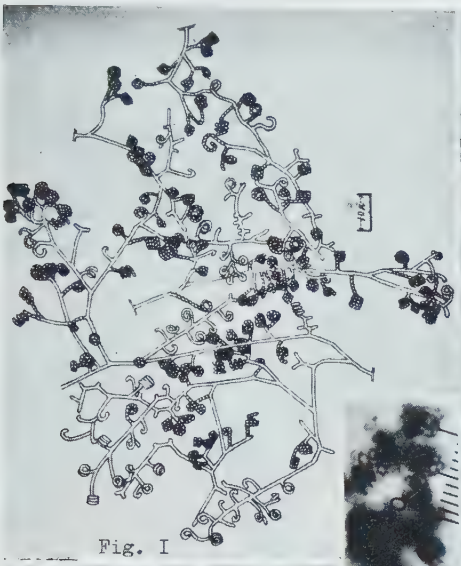
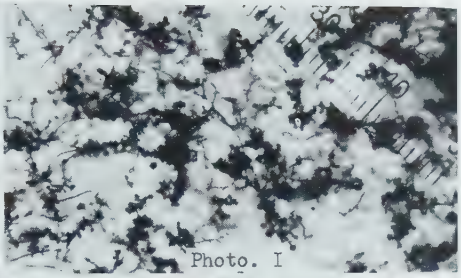


Fig. I



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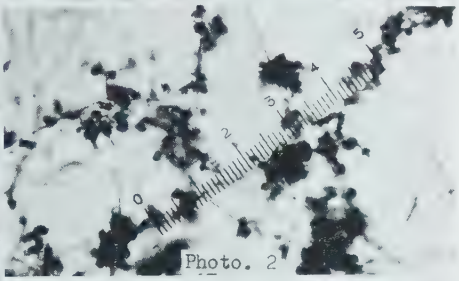

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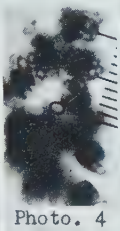

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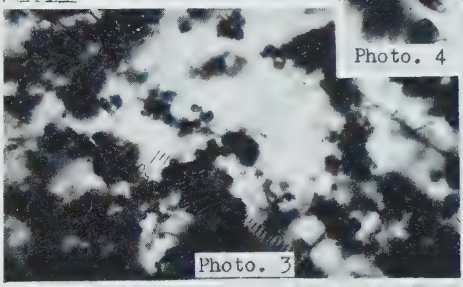

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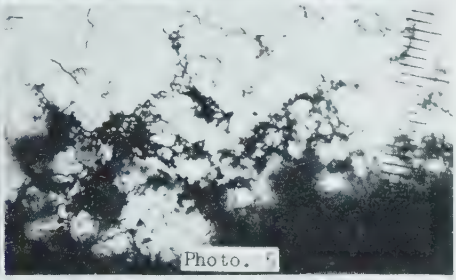

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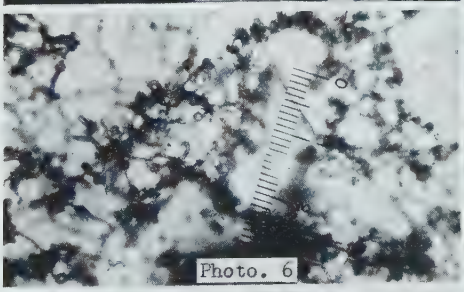


Photo. 6

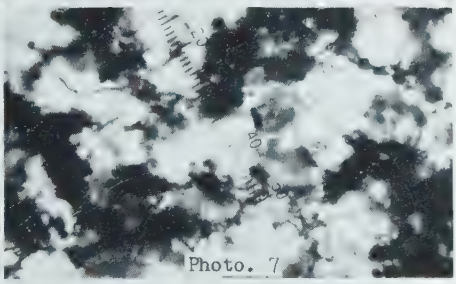
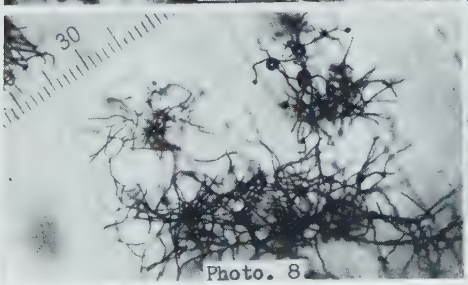


Photo. 7



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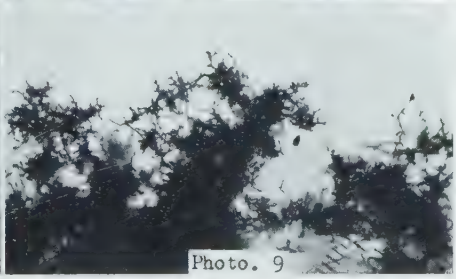

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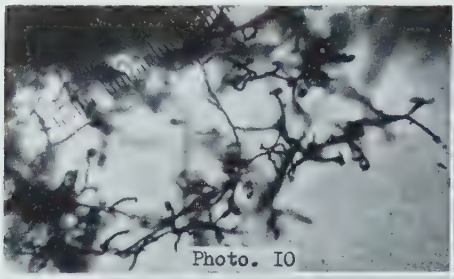
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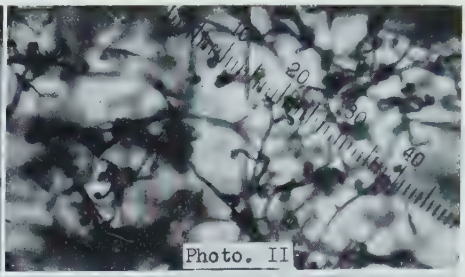
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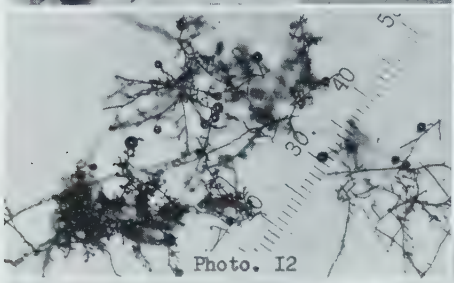
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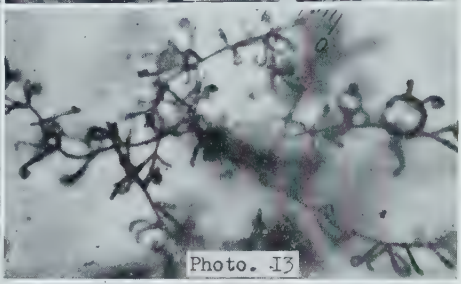
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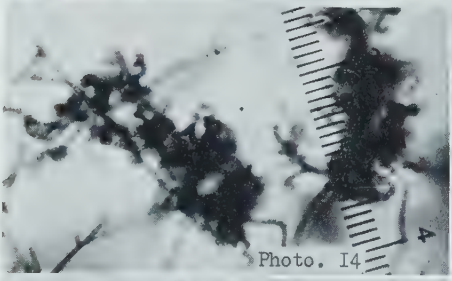
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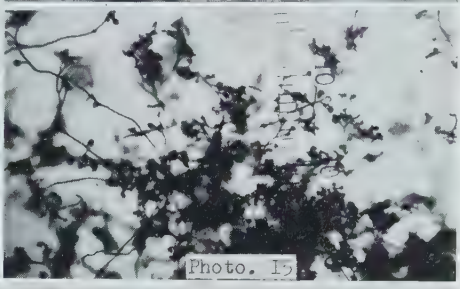
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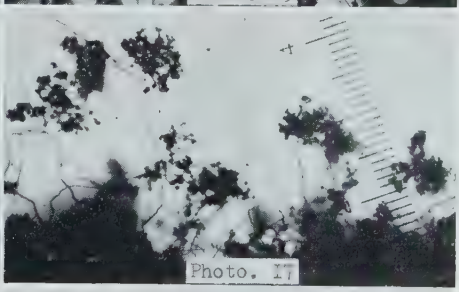
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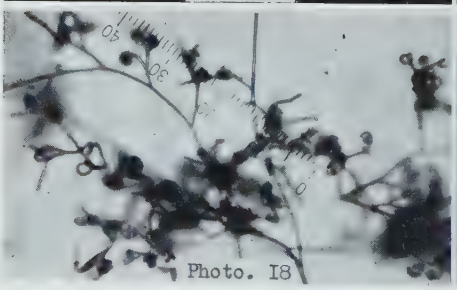
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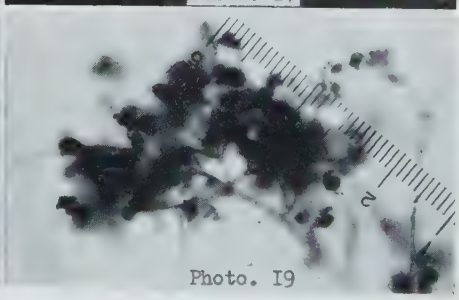
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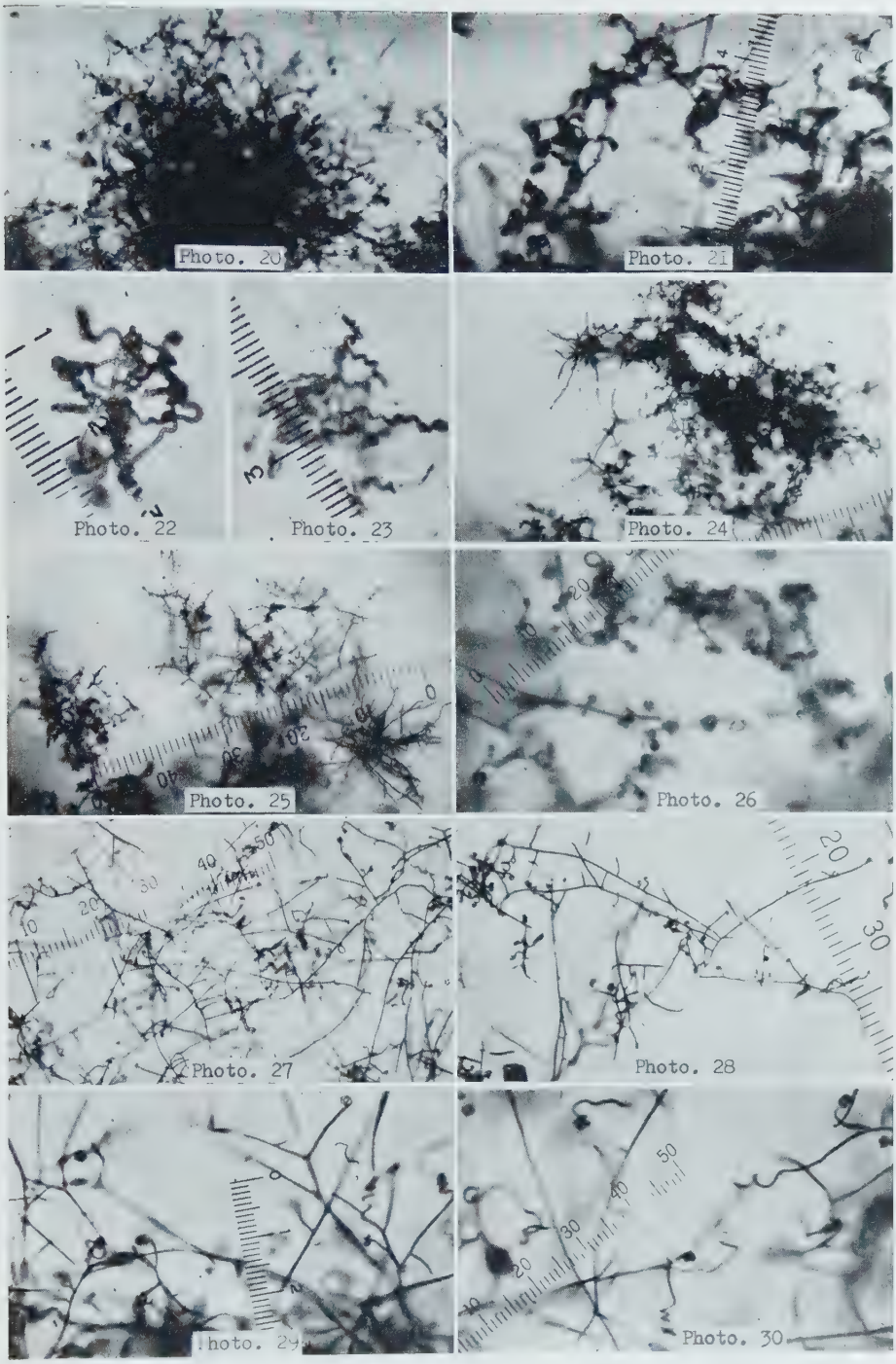
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STUDIES ON THE CLASSIFICATION OF STREPTOMYCETES, PART XIII THE MICROSCOPIC MORPHOLOGY AND OTHER CHARACTERISTICS OF WHORL FORMING STRAINS

RYOSAKU NOMI

Faculty of Engineering, University of Hiroshima

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On the whorl-forming strains many reports have been published and the microscopic study has also been described considerably in detail (*1*~*4*). In this paper the author presents more detailed microscopic characteristics of related strains.

Although the definition of the term " whorl " is not necessarily distinct, it may probably mean verticillate arrangement of the sporiferous hyphae. The author uses the term " whorl " restrictively in the case of branching form of the sporiferous part, though verticillate form of branching also exists in the case of branching of non-sporiferous mycelia in various strains.

The " whorl-form " in sporiferous part is very distinct and characteristic in some species, but in whorl-forming strains the morphology of non-sporiferous part is also characteristic, and further there may exist a little deformed whorl form of sporiferous part in some species. The author describes the details about them in the following.

EXPERIMENTS AND RESULTS

I. Strains Employed in this Study

The strains employed are *Streptomyces reticuli* HUT 6028 [←IFO 3354], *Streptomyces griseocarneus* HUT 6055 [←IAM W3-5←IID←NRRL B-1068], *Streptomyces echimensis* HUT 6042 [←IAM W7-4←IFO 3398], *Streptomyces albireticuli* HUT 6040 [←IAM W7-6←IFO 3400], *Streptomyces thioluteus* HUT 6071 [←IAM W6-5←NIHJ 26A], *Streptomyces hiroshimensis* HUT 6033 [←Shinobu, Osaka Univ. of Liberal Arts and Education, Japan], *Streptomyces salmonicida* HUT 6085 [←IAM←NRRL B-1472] and *Streptomyces netropsis* HUT 6086 [←IAM←NRRL 2268].

II. Experimental Method and Items

Experimental method and items are the same as in the preceding paper (*5*). Microscopic morphology was observed using direct observation method of Petri plate culture as well as cover culture method (*6*). The slant

cultural features on Czapek-Dox agar, Krainsky agar, Bennett agar and modified Bennett agar, and starch hydrolysis test and behavior on skim-milk and nitrate reduction test of each strain employed were studied and recorded. Incubation was carried out for fourteen days at 28–30°C, with the exception of seven days incubation in starch hydrolysis test. The data are given at the end of this paper under "Description of Strain".

When cultural medium is prepared, pH value is prescribed, in many case, to be adjusted at neutral or slightly basic point. In special case, adjustment at above 8.0 is needed. But the author considers that it is very useful in morphological study to pay more attention to pH value of cultural media.

Generally speaking, *Streptomyces* has a considerably distinct tendency to produce acid from carbon sources and ammonium from nitrogen sources. In an early stage of growth, acid formation exceeds ammonium formation, resulting in the decrease of pH value which can be detected by putting pH test paper on the slant agar. It is not uncommon to decrease to about pH 5 from initial pH 7 in Bennett agar. In accordance with the development of growth, pH value increases gradually, namely ammonium formation increases to exceed acid formation and finally reaches somewhere about 8.5 (8.0 to 9.0) in many cultures. In such case, if a slant culture is used, ammonium gas accumulates in the test tube, which is detectable with filter paper moistened with Nessler's reagent. While such phenomenon is commonly observed in organic medium such as Bennett agar, it is often observable also in synthetic medium such as Czapek-Dox agar for instance. When the pH value of cultural medium reached somewhere about 8.5, many cultures either stop their subsequent morphological growth or will have finished their growth by that time. In such cultures, the initial pH may well be lower than 7.

However, there are some strains whose acid formation is considered to be more vigorous than their deamination. When such strains grow on Bennett agar, the final pH value comes to a halt at about 7, and sometimes it becomes lower than initial pH. In such strains, initial pH had better be adjusted at 8 to 9. One of these strains is *Streptomyces caeruleus*, and among the strains studied *S. thioluteus* is the one which falls under the same category, while *S. echimensis* shows the same tendency.

When a cultural medium is prepared, method of pH adjustment is important, especially when the pH value needs to be raised. Even though the pH value is adjusted at 8.5 in Bennett agar before sterilization, it will decrease almost down to 7 in sterilization process (15 lb., 15 min.). This is probably due to agar reaction. Therefore, when pH value is desired to be brought to above 8.0, it must be adjusted after sterilization and before using (e. g. before slanting) aseptically.

The pH value of cultural medium is not constant through the all stages of growth of strain but it is changeable. This circumstances are easily understood from the above-mentioned information. Now, the progress of pH value of cultural medium is principally influenced by the ratio of carbon

source/nitrogen source in medium. A large C/N ratio has a tendency to make pH value lower in the growth of strain, owing to excessive acid formation. On the contrary, when the C/N ratio is small, the tendency is reversed. Therefore, when the high initial pH is required, the lower C/N ratio in the medium may be substituted for pH adjustment, namely the quantity of carbon source may well be decreased. Contrary, the high C/N ratio may be substituted for adjustment at lower initial pH value. But the change in the quantity of carbon source may influence the amount of the aerial and vegetative mycelium.

III. General Microscopic Morphology

The strains employed in this study have no spirals.

Generally, all strains employed have long straight or long wavy axial aerial mycelia which branch repeatedly in monopodial alternate, opposite and verticillate forms, and occasionally diverge from one filament into two or three branches. This branching system is the same as that of *Streptomyces sp.* No. 240 (*7*) and No. 477 (*8*) which are spiral forming strains reported in previous papers. Long wavy mycelium may be somewhat aged form of long straight mycelium. The long axial aerial mycelium does not break into oidiospores, but gives rise to short side branches which grow in verticils and form whorls. The terminal hyphae of whorl sporulate.

In taxonomy of Pridham et al., four types of verticillate sporiferous parts are described, i. e. Monoverticillus, Monoverticillus-Spira, Biverticillus and Biverticillus-Spira. About three types among them, they state as follows; " three sections (Monoverticillus, Monoverticillus-Spira and Biverticillus-Spira) are included to develop an orderly morphological system for cataloging strains. Although our collection of Streptomycetes contains no isolates that could be placed with certainty in any of these sections, there are allusions to these morphological types in the literature. In our opinion, these sections have no valid taxonomic status at present. While this treatment may seem somewhat presumptuous we believe that their inclusion will assist in clarifying some of the problems that arise in any discussion of taxonomic system for the Streptomycetes ".

Also the author does not know such strains in which Monoverticillus-Spira or Biverticillus-Spira is solely the main branching form of sporiferous part. According to author's study, these two branching systems are often contained in various spiral forming strains, but they are not contained so uniformly as they warrant taxonomic criteria. And in such spiral forming strains verticillate branching systems are contained not only in the sporiferous parts, but also in other branching parts of aerial mycelia, and furthermore, they are not always attached to long axial mycelia. Therefore the author considers that MV-S and BIV-S are but a special and exceptional case of verticillate branching form in the various monopodial branching forms of aerial mycelia in spiral forming strains, and it is not necessarily appropriate to let these two branching forms belong to whorl form.

On the other hand, Monoverticillus is considered to be competent for being counted as a case of whorl form.

The whorl form may principally be classified as the typical and atypical form. The typical whorl form corresponds to Biverticillus defined by PRIDHAM et al. In many typical whorl forming strains short side braches are compounded of primary and secondary elements. The primary whorl is verticillated from long axial mycelium, namely two to six short side branches are projected outwards radially from a point of axial mycelium. Secondary whorl is projected outwards from each apex of primary whorl into an umbellate form. Number of the umbellate branches is about ten or so at its maximum. In this compounded whorl the sporiferous part is only the secondary whorl and the primary whorl does not sporulate. The interval of whorls on a long axial aerail mycelium is not uniform.

Occasionally, one filament of primary whorl continues its growth without bearing secondary whorl, resulting to formation of a long straight mycelium which gives rise again to whorls as side branches.

While the above-mentioned whorl is typical whorl, there are some atypical whorls which are contained in large number in *Streptomyces netropsis* and considerably so in *Streptomyces albireticuli* and sometimes in typical whorl forming strains. The forms of atypical whorls are various. The above-mentioned Monoverticillus is one of them, and sometimes some components of verticils in Monoverticillus give rise to secondary whorls, leaving other components just as they are. In such case, while the components corresponding to Monoverticillus sporulate entirely, the components bearing secondary whorls sporulate only at the secondary elements.

Also sometimes, a short side branch arisen from a long axial mycelium bears two to three or more steps of whorl and furthermore several of such steps' whorl-bearing short side branches are possibly verticillated from a point of a long axial mycelium.

It is not uncommon that single short side branches arise plentifully and thickly from axial mycelia and break into spores.

In such atypical whorl forming strains, the interval of whorls is more irregular and the long axial mycelium is generally shorter than those of the typical whorl forming strains. Furthermore, in some part of colony, various whorls including many atypical whorls thickly arise at short intervals on a axial mycelium. In such part, the branching system appears different from whorl form, and really, the branching system of side branches is not always verticils but sometimes alternate or opposite forms. Though such exceptional branching system is also included in atypical whorl forming strains, the author cannot help letting the strain to be held in whorl forming strain, regarding, as he does, the exceptional branching forms as incomplete and abnormal forms.

As a result, the microscopic appearance of the atypical whorl forming strain often looks in a jumble, as compared with the typical whorl forming strain.

Atypical whorls are sometimes contained in the typical whorl forming strain and their contained degree is changeable depending on the strain and cultural condition. When cultural condition is unfavorable, it is common that atypical whorls increase than they do in the case of favourable cultural condition. But, in general, sporulation of such atypical whorls in the typical whorl forming strain is often incomplete or elso does not occur.

The terminal sporulated filaments of both typical and atypical whorls are straight or a little wavy, and are generally 4.5 to 20 μ in length while they develop occasionally even up to about 45 μ in the atypical whorl.

In the typical whorl forming strain, when well developed, primary elements of whorl occasionally tapers down and their tips are a little swollen. But as occurrence of such tapering and swelling is not constantly repetitive, it is difficult to determine whether or not the characteristics is valid as taxonomic criterion.

DESCRIPTION OF STRAIN

In the following description, (A), (V), (R) and (P) mean aerial mycelium, vegetative mycelium, reverse of colony and soluble pigment, respectively. On the aerial mycelium the degree of growth is expressed in terms of five grades; non, slight, moderate, fairly good and good. Italic color name is quoted from RIDGWAY's Color Standards and Nomenclature 1912. In modified Bennett agar, favourable carbon sources are shown in round brackets. The name of slant cultural medium is curtailed " agar ".

The following description may contain some difference from original description. But it is difficult to determine whether it is due to degeneration of the culture among preservation or to other unknown circumstances.

***Streptomyces reticuli* HUT 6028**

I. Whorl is principally typical. **Spore:** short rod (occasionally ellipsoid), 0.6 to 0.8 by 0.7 to 1.3μ.

II. Czapek: (A) good; white to *Pale Pinkish Cinnamon*. (R) brownish gray to grayish brown. (P) almost non. **Krainsky:** (A) good; grayish white. (R) deep dark brown. (P) non. **Bennett:** (A) good; *Tilleul Buff* to a little pale *Vinaceous-Buff* or (*Pale* to) *Light Vinaceous-Fawn* (R) dark yellowish brown. (P) dirty pale yellow. **Mod. Bennett** (glycerol, maltose and starch): (A) good; (*Light* to) *Pale Pinkish Cinnamon* to (*Pale* to) *Light Vinaceous-Fawn*. (R) dark yellowish brown to dirty brown. (P) almost non (maltose and starch) to pale yellowish brown (glycerol).

Starch: Hydrolysis positive. **Milk:** Both coagulation and peptonization negative; grayish brown soluble pigment. **Nitrate:** Reduction negative.

***Streptomyces hiroshimensis* HUT 6033**

I. Whorl is principally typical. **Spore:** ellipsoid to short rod, 0.7 to 0.9 by 0.8 to 1.5μ.

II. Czapek: (A) fairly good; white to partially *Hermosa Pink*. (R) red (*Old Rose* to *Light Jasper Red*). (P) slightly yellow. **Krainsky:** (A) good; white to partially *Salmon-Buff*. (R) dark red (*Old Rose* to *Acajou Red* or *Vandyke Red*). (P) non. **Bennett:** (A) good; *Pale Ochraceous-Buff* to intermediate color between *Shell Pink* and *Buff-Pink*. (R) deep dark red. (P) dirty brown. **Mod. Bennett** (maltose and starch): (A) good; almost same to the color in Bennett agar. (R) deep dark red. (P) dirty brownish yellow.

Starch: Hydrolysis positive. **Milk:** Both coagulation and peptonization positive; deep brownish black soluble pigment. **Nitrate:** Reduction positive.

***Streptomyces albireticuli* HUT 6040**

I. Whorl contains fairly many atypical forms together with typical form. **Spore:** short rod; 0.5 to 0.8 by 0.8 to 1.5μ.

II. Czapek: no growth. **Krainsky:** (A) good; white to slightly yellow or yellowish white. (R) pale yellow. (P) non. **Bennett:** (A) fairly good; white. (R) yellowish brown→brown. (P) dirty yellow→brownish yellow. **Mod. Bennett** (maltose and starch): (A) good; white. (R) dirty yellowish brown→brown. (P) dirty yellow→dirty yellowish brown.

Starch: Hydrolysis positive. **Milk:** Both coagulation and peptonization slight; deep dark brown soluble pigment. **Nitrate:** Reduction positive.

***Streptomyces echimensis* HUT 6042**

I. Whorl is principally typical. **Spore:** short rod, 0.5 to 0.8 by 0.8 to 2.0μ.

II. Czapek: (A) slight; yellowish white to pale yellow. (R) yellowish brown or dirty orange. (P) almost non to slightly yellow. **Krainsky:** (A) moderate to fairly good; white to *Light Pinkish Cinnamon*. (R) pale yellow to brownish yellow. (P) almost non. **Bennet:** (A) good; white to brownish white. (R) orange to yellowish brown. (P) dirty yellow. **Mod. Bennett** (maltose and starch): (A) good; (*Pale* to) *Light Vinaceous-Fawn* to *Vinaceous-Buff*. (R) dirty orange to yellowish brown to brown. (P) yellowish orange to brownish yellow.

Starch: Hydrolysis positive. **Milk:** Both coagulation and peptonization positive; orange to yellowish brown soluble pigment. **Nitrate:** Reduction negative.

***Streptomyces griseocarneus* HUT 6055**

This strain seems to be closely related with *Streptomyces reticuli* HUT 6028.

I. Whorl is principally typical, but the ability to form aerial mycelium seems to be a little degenerated. **Spore:** short rod, 0.5 to 0.8 by 0.7 to 1.3μ.

II. Czapek: (A) moderate; white. (R) dark yellowish brown. (P) pale dirty yellow. **Krainsky:** (A) fairly good; grayish white. (R) deep dark brown. (P) non. **Bennett:** (A) moderate; dirty white or slightly gray. (R) dark brown. (P) slightly dirty yellow. **Mod. Bennett** (maltose and starch):

(A) fairly good; (*Pale* to) *Light Pinkish Cinnamon*. (R) dark brown. (P) almost non to slightly dirty yellow.

Starch: Hydrolysis positive. **Milk:** Both coagulation and peptonization negative; grayish brown soluble pigment. **Nitrate:** Reduction negative.

***Streptomyces thioluteus* HUT 6071**

I. This strain is a little alkaliphile for the favourable development of aerial mycelium. The favourable pH value in Bennett agar is about 8. Although whorl is surely born, the author cannot decide with certainty whether the whorl is typical or atypical, because whorl formation is not successfully observed in sufficient instances. According to observation of a few whorls, however, it is probably typical whorl. **Spore:** short rod, 0.4 to 0.8 by 0.8 to 2.0μ.

II. Czapek: growth is hard to attain. But, once the growth is set in, it develops slowly. (A) fairly good; *Dark Olive-Buff*, powdery to dirty white, cottony. (R) deep dark brown. (P) brown. **Krainsky:** (A) moderate; dirty yellowish white. Fine reddish orange crystals are produced in abundance on the aerial mycelium. (R) dark yellowish brown. (P) dirty yellow and brown. **Bennett:** (A) moderate; *Olive-Buff*, powdery. Fine reddish orange (*Ochraceous-Orange*) crystals are much produced on the aerial mycelium. When pH value is adjusted at 8: (A) good; (*Pale* to) *Olive-Buff*, cottony. Reddish orange crystals are not produced. (R) orange→deep dark brown. (P) yellow →yellow and brown (both fairly concentrated). **Mod. Bennett** (maltose): (A) fairly good; (*Deep* to) *Olive-Buff*, cottony. Almost no crystals. (R) brown. (P) fairly deep yellow.

Starch: No growth on ammonium sulfate medium. On the plate of starch-Bennet agar and starch-Krainsky agar, starch is not hydrolyzed. **Milk:** Coagulation positive,no peptonization. **Nitrate:** Reduction negative.

***Streptomyces salmonicida* HUT 6085**

I. Whorl is principally typical. **Spore:** short rod, 0.5 to 0.8 by 0.8 to 1.5μ.

II. Czapek: (A) slight; white. (R) red (*Carnelian Red* to *Dragon's-blood Red*). (P) yellow and brown. **Krainsky:** (A) good; *Cartridge Buff*. (R) red (*Coral Pink* to *Coral Red*). (P) yellow. **Bennett:** (A) good; *Pale Olive-Buff* to *Colonial Buff*. (R) dark red (*Brick Red* to *Hay's Russet*). (P) yellow to yellowish orange to yellowish brown. **Mod. Bennett** (maltose and starch): almost same to the case of Bennett agar.

Starch: Hydrolysis positive. **Milk:** Both coagulation and peptonization negative; grayish brown soluble pigment. **Nitrate:** Reduction positive.

***Streptomyces netropsis* HUT 6086**

I. Whorl contains many atypical forms. **Spore:** short rod, 0.5 to 0.8 by 0.6 to 1.3μ.

II. Czapek: No growth. **Krainsky:** (A) slight; *Vinaceous-Buff* to *Avel-*

laneous. (R) deep dark brown. (P) brown. **Bennett:** (A) moderate; *Pale Congo Pink, Light Vinaceous-Fawn, Pale Grayish Vinaceous* and *Vinaceous-Pink*. (R) deep blackish brown to brownish black. (P) brown. **Mod. Bennett** (glycerol, fructose and starch): (A) good; *Light Pinkish Cinnamon, Light Vinaceous-Cinnamon, Light Vinaceous-Fawn* and *Pale Grayish Vinaceous*. (R) brownish red to brownish black (fructose and starch) or brownish black (glycerol). (P) dirty red (fructose and starch) or brown (glycerol).

Starch: Hydrolysis positive. **Milk:** No coagulation, peptonization positive. **Nitrate:** Reduction negative.

SUMMARY

The whorl forming strains have long straight or long wavy axial aerial mycelia which branch repeatedly in monopodial alternate, opposite and verticillate forms, and occasionally diverge from a filament into two or three branches. The long axial aerial mycelium does not break into spores, but gives rise to short side branches which grow in verticils and form whorls. The typical whorl is "Biverticils", namely compound verticils consisting of primary and secondary elements. And there are sometimes atypical whorls in some unfavourable cultural condition or in some strains. Atypical whorls contain "Monoverticils" consisting of only primary verticils, mixed verticils of mono- and bi-verticils and sometimes various more compound polyverticils.

The terminal hyphae of whorl are short straight or a little wavy in shape and break into spores.

The whorl forming strains employed have no spirals.

The description of each strain employed was also given.

The author wishes to express his sincere thanks to Dr. K. SAKAGUCHI for his guidance and Dr. T. NEHIRA, Dr. K. ARIMA and Dr. Y. IKEDA for their encouragement. The author is indebted to Mr. T. HASEGAWA, Dr. H. IIZUKA and Dr. R. SHINOBU for their kindness in supplying strains used in this study. This work was supported in part by Grant for Co-Operative Research from the Ministry of Education and in part by Researching Subsidy from Waksman Foundation in Japan.

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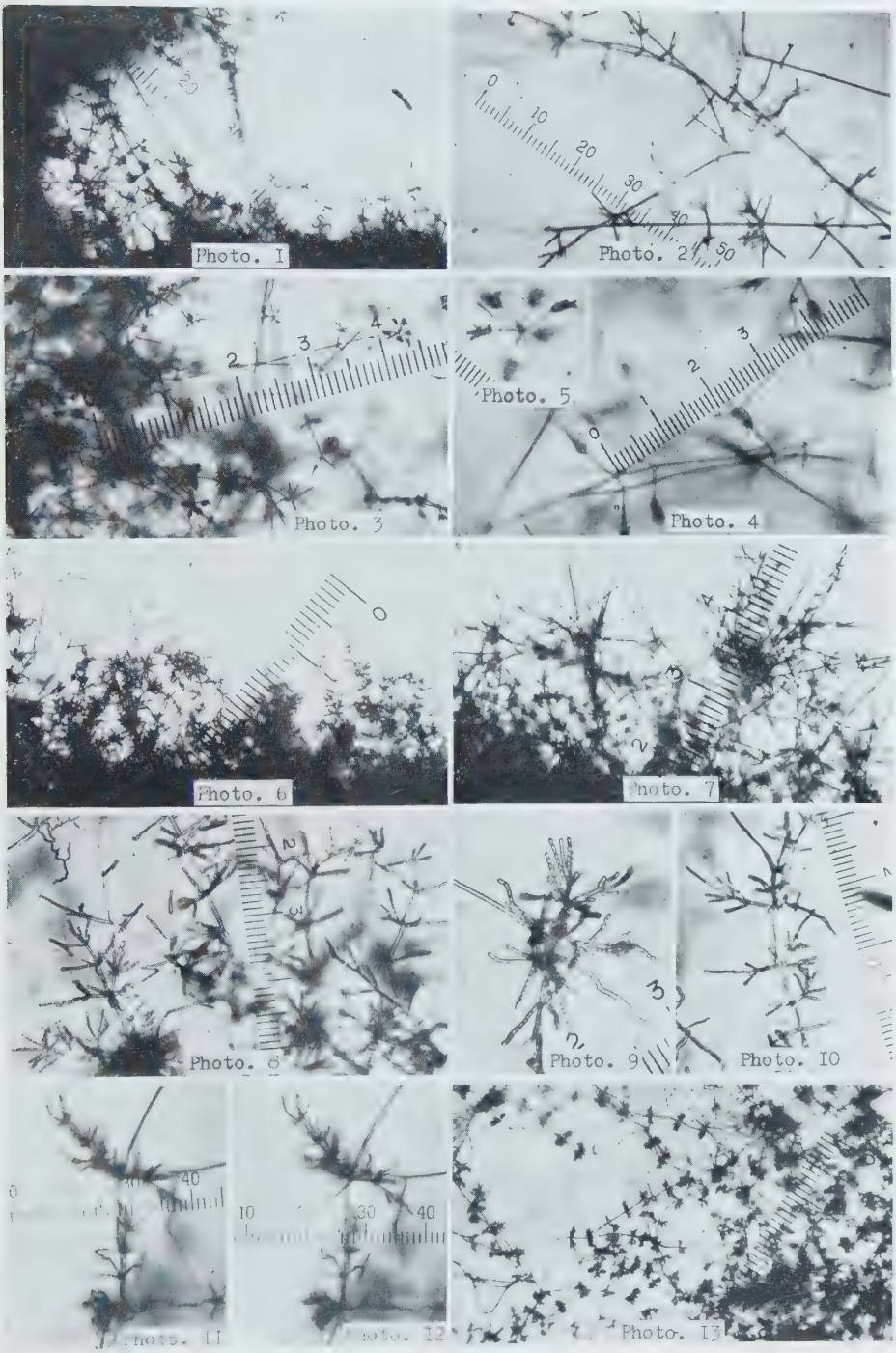
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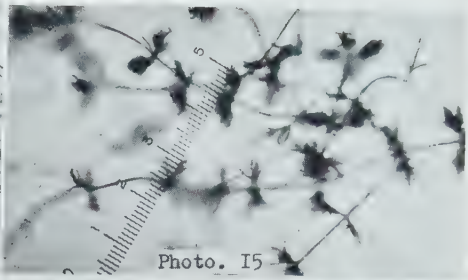
Photo. I4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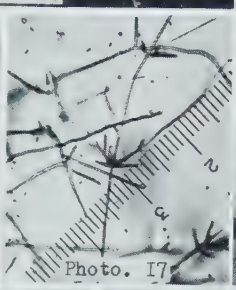
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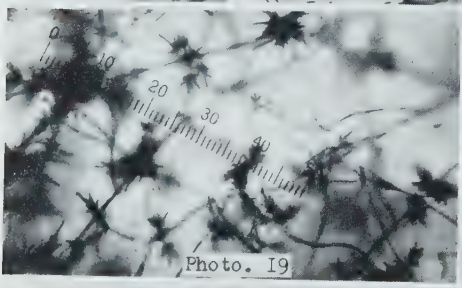
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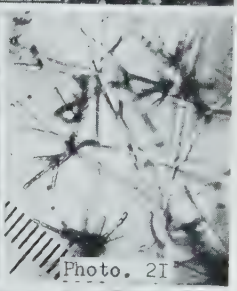
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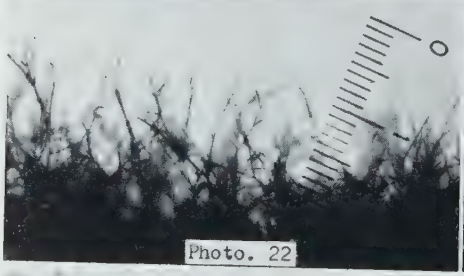
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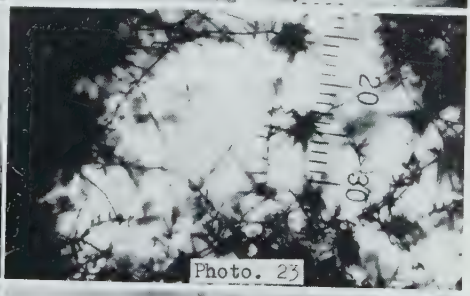
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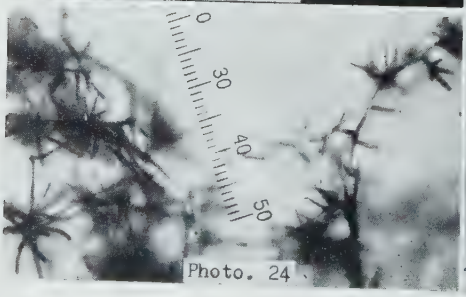
Photo. 24

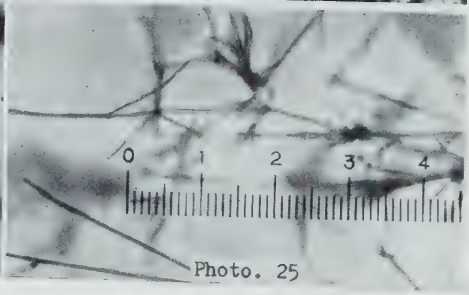
Photo. 25

(*6*) H. NAGANISHI and R. NOMI: *J. Ferment. Techn.* Japan, **31**, 476 (1953); R. NOMI: *ibid.*, **33**, 341 (1955).
(*7*) H. NAGANISHI and R. NOMI: *J. Ferment. Techn.* Japan, **32**, 492 (1954).
(*8*) *ibid..*, **33**, 294 (1954).

EXPLANATION OF PLATES IV–V

Photos. 1 and 2 are *S. reticuli* HUT 6028. Photo 1 (100× microscopic magnification) and Photo. 2 (450× microscopic magnification) show a part of colony.

Photos. 3–5 are *S. echimensis* HUT 6042. Photo. 3 (100×) and Photo. 4 (450×) show a part of colony. Photo. 5 (450×) shows a case of typical whorl.

Photos. 6–12 are *S. albireticuli* HUT 6040. Photo. 6 (100×) shows a part of colony where well developed typical and atypical whorls are attached to axial mycelia. Photo. 7 (100×) shows young stage of growth of long straight axial mycelia where whorls are not yet attached in abundance. Photos. 8–10 (all 450×) show various forms of whorl attached to axial mycelia and sporulation of terminal elements of whorls. Photos. 11 and 12 (both 450×) show an atypical whorl and these are the photographs of the same object; focuses were set a little apart from each other.

Photos. 13–17 are *S. hiroshimensis* HUT 6033. Photos. 13, 14 (both 100×) and 15 (450×) show a part of colony where many typical whorls are attached to axial mycelia. Photo. 16 (450×) shows sporulation of secondary elements of typical whorl. Photo. 17 (450×) shows that a secondary element of whorl may grow on resulting in formation of a long straight axial mycelium.

Photos. 18–21 are *S. salmonicida* HUT 6085. Photo. 18 (100×) and Photo. 19 (450×) show a part of colony where many typical whorls are attached to axial mycelia. Photos. 20 and 21 (both 450×) show sporulation of whorls.

Photos. 22–25 are *S. netropsis* HUT 6086. Photo. 22 (100×) shows axial mycelia before whorls are formed. Photo. 23 (100×) shows a part of colony after whorls were attached to axial mycelia. Photos. 24 and 25 (both 450×) show many atypical and a few typical whorls attached to axial mycelia.

One unit of the scale in the photographs is 11 μ in 100×'s photographs and 2.5 μ in 450×'s photographs.

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A TAXONOMIC STUDY ON THE GENUS *RHODOTORULA*

1. THE SUBGENUS *RUBROTORULA* NOV. SUBGEN.

TAKEZI HASEGAWA, ISAO BANNO and SAKAE YAMAUCHI

Institute for Fermentation, Osaka

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As to the genus *Rhodotorula*, the first systematic study was attained by SAITO (*1*) in 1922. He classified fourteen red- and yellow-colored *Torulas* by the nature of clony color, the morphology of cells, the assimilability of sugars and nitrate, and by the ability of gelatin liquefaction. Later OKUNUKI (*2*) also studied on the colored *Torula* in 1931 and added six new species to the above results. The names of the *Torula* yeasts by SAITO and by OKUNUKI are as follows:

Yellow *Torulas*: *Torula luteola*, *T. gelatinosa*, *T. aurea*, *T. flavescens*, *T. flava*, *T. rugosa* (by SAITO)

Red *Torulas*: ? *T. sanguinea*, *T. rufula*, *T. corallina* ? *T. rubra*, *T. rubra*, var. *α*, *T. minuta* *T. ramosa*, *T. rubescens*, *T. aurantiaca* (by SAITO) *T. suganii*, *T. infirmominiata*, *T. miniata* *T. decolans*, *T. koishikawensis*, *T. shibatana* (by OKUNUKI)

HARRISON (1928) (*3*) divided these colored yeasts into *Rhodotorula* and *Chromotorula* based on the color of colony. LODDER (*4*), in her taxonomic monograph "Anascosporogenen Hefen" (1934), brought all the asporogenous yeasts producing carotenoid pigments into the genus *Rhodotorula* including 13 species and 10 varieties. In 1952, LODDER and KREGER-VAN RIJ (*5*) rearranged the genus, which was classified into the following seven species and one variety.

Rhodotorula glutinis (FRES.) HARRISON
Rhodotorula glutinis var. *rubescens* (SAITO) LODDER et KREGER-VAN RIJ
Rhodotorula aurantiaca (SAITO) LODDER
Rhodotoula pallida LODDER
Rhodotorula minuta (SAITO) HARRISON
Rhodotorula rubra (DEMME) LODDER
Rhodotorula mucilaginosa (JÖRG.) HARRISON
Rhodotorula flava (SAITO) LODDER

The species and variety were discriminated each other by the assimilabilities of sugars and potassium nitrate and the shape of cells, but the differentiation by means of the colony color was not employed for the reason that these were nothing but the results of variable ratio in the composition

of carotenoid pigments produced in cells. In the work, there were many marked discrepancies between the descriptions of the shape of cells by the authors and by the original authors. Doubtful points about this were reported by HASEGAWA in 1956 (*6*). He had investigated the morphological and physiological properties of 28 cultures of 19 *Rhodotorula* which included several groups, each of which consisted of 2 or 3 lineal descendants of SAITO's or OKUNUKI's strain. He stated that the shape of cells in nine cultures among them did not agree with the description by LODDER and KREGER-VAN RIJ, although that of four in the nine cultures coincided well with that by the original authors and that besides, in some descendant groups, the cultures differed each other as to the shape of cells.

Furthermore, his study was continued. He surveyed forty-six *Rhodotorula* cultures descended from twelve of SAITO's and OKUNUKI's strains which had been maintained in separate organs in Japan, and the following conclusions were reached (*7*).

1) The natural variation in cell morphology just resulted in the discrepancy between the description by original authors and that by LODDER and KREGER-VAN RIJ. In the latter system, therefore, the differentiation of the species and variety by the shape of cells is invalid; *Rhodotorula aurantiaca* and *Rht. glutinis* var. *rubescens* are synonymous with *Rht. glutinis*, and *Rht. mucilaginosa* with *Rht. rubra*.

2) With the exception of *Torula aurantiaca* and *T. flava*, all the cultures are similar in the colony color ranging from yellowish red to orange*, and the color of *T. aurantiaca* is yellowish orange, that of *T. flava* is reddish yellow. A taxonomic distinction should be made between these two and the common *Rhodotorulas*.

(In the work above mentioned, HASEGAWA regarded *Rhodotorula flava* as a variety of *Rhodotorula tokyoensis*, but the conclusion was misleading. As the details will be explained later in this paper, *Rhodotorula flava* and *Rhodotorula tokyoensis* are two entirely different species.)

After the publication of the LODDER and KREGER-VAN RIJ's taxonomic work, the following new species and new variety were reported.

Rhodotorula marina PHAFF, MRAK et WILLIAMS (1952) (*8*)
Rhodotorula texensis PHAFF, MRAK et WILLIAMS (1952) (*8*)
Rhodotorula peneaus PHAFF, MRAK et WILLIAMS (1952) (*8*)
Rhodotorula tokyoensis KOBAYASHI (1953) (*9*)
Rhodotorula crocea SHIFRINE et PHAFF (1956) (*10*)
Rhodotorula macerans SONNE FREDERIKSEN (*11*)
Rhodotorula glutinis (FRFS.) HARRISON var. *dairenensis* HASEGAWA et BANNO (1958) (*12*)
Rhodotorula lactosa HASEGAWA (1959) (*13*)

* The colors were named according to "Guide To Color Standard" published by the Nihon Shikisai Kenkyusho in 1954, which was based on National Bureau of Standard: ISCC–N.B.S. Color Name 23 (1939).

The SAITO's yellow *Torulas* except *Torula flava* were classified into the genus *Cryptococcus* by LODDER and KREGER-VAN RIJ. According to these authors, *Cryptococcus* was a genus distinguished from *Torulopsis* by the cells capsulated as well as by the mucous appearance, and from *Rhodotorula* by the extracellular starch formation and by the lack of carotenoid pigments. But recently, several examples which led to internal disorder between *Cryptococcus* and *Rhodotorula* have been reported. Formerly, both *Cryptococcus* and *Rhodotorula* were treated as the same genus (*14*). WICKERHAM suggested in his paper (15) that the starch production would not serve well as a principal characteristic in separation of genera. Indeed, *Rhodotorulas* producing starch were found. These are *Rht. peneaus* (*8*), *Rht. macerans* (*11*), *Rht. glutinis* var. *infirmominiata* etc. (*16*). On the other hand, NAKAYAMA *et al* (*17*) and PETERSON *et al* (*18*) reported the carotenogenesis in several strains belonging to *Cryptococcus*.

However, this does not detract in the least the value of the brilliant work of LODDER and KREGER-VAN RIJ which brought a wonderful simplification into the taxonomic system of the yeasts. Our studies about the vitamin requirement of *Rhodotorula* and *Cryptococcus* resulted in the discovery of a very interesting relationship with their taxonomic properties, and consequently of a clue to the phylogenetic interrelationship of the species.

We wish to inform of the particulars noted above, and a new taxonomic system of the genus *Rhodotorula* will be discussed in this report.

1. Taxonomic significance of the color due to carotenoid pigments

In 1922, SAITO (*1*) divided Torula yeasts into the three groups with the color of colony; white to gray white, yellowish gray to yellow and orange to red. Later, HARRISON (*3*) created the genera *Rhodotorula* and *Chromotorula* for the colored asporogenous yeasts; the former for the red yeasts, the latter for those other than red, i.e. yellow, brown or black yeasts.

But, in 1934, LODDER (*4*) pointed out the fact that it was hardly possible to make a separation among red, yellow red, orange and yellow colored yeasts. Moreover, the black yeasts placed in *Chromotorula* deviate markedly in morphological aspects from the other forms assigned to the group. She, therefore brought all the asporogenous yeasts with carotenoid pigments into the genus *Rhodotorula* and rejected the genus *Chromotorula*.

As to the existence of carotenoid pigments in *Rhodotorula* yeasts, the first report was published by ZOPF in 1889 (*19*). He noted the presence of lipochromes, but nothing further had been reported until LEDERER (*20*) proved in *Rht. rubra* four pigments; an acidic pigment, β-carotene, torulene and an undefined pigment. KARRER and RUTSCHMANN (*21*) investigated the acidic pigment and named it torularhodin. Thereafter, γ-carotene (*22*), neurosporene (*23*), phytofluene (*22*) and lycopene (*17*) were also found in the yeast.

PHAFF *et al* (*8*) observed an interesting phenomenon that some strains of *Rht. glutinis* were red when they had grown at room temperature but

appeared yellowish at lower temperature. Further examinations into this were carried out by NAKAYAMA *et al* (*17*). They used a strain of *Rht. rubra* which remained red when grown at 5°C. and a strain of *Rht. peneaus*, a yellow *Rhodotorula* which had a paler color at low temperature. When comparison was made in *Rht. peneaus* at the two incubation temperatures, 25°C and 5°C, the total pigments and β-carotene concentrations showed a marked drop at the lower temperature. The carotenoids of *Rht. rubra* were relatively little modified by the alteration of temperature, whereas in *Rht. glutinis* at 25°C, the yellow carotenoids; β- and γ-carotene comprise 43–47% and the red carotenoids; torulene and torularhodin 53-57% of the total pigments, yet, at 5°C, the yellow carotenoids 92–96% and the red carotenoids only 4–8%. In the case of lower temperature, the absorption spectrum in petroleum ether of the total pigment of *Rht. glutinis* coincided with that of *Rht. peneaus* at 25°C, differing from that of *Rht. glutinis* at 25°C.

According to PETERSON *et al* (*18*), the absorption maxima of petroleum ether extracts of the *Rhodotorulas* were at 480 mμ or 480 mμ and 450 mμ, or 450 mμ, but those of the yellow *Rhodotorula* and *Cryptococcus* at 450 mμ.

WITTMANN (24) pointed out that the composition of carotenoids in *Rht. rubra* was changeable with the components of culture media.

Our results accorded with the above observations. Thirty cultures of the *red Rhodotorulas* and three cultures of yellow *Rhodotorulas* were compared on the carotenogenesis in the WICKERHAM's synthetic medium containing vitamins (*25*) (*26*).

As to the *Rhodotorulas* the absorption maxima of extracts of six cultures were at 450 mμ and these of twenty three cultures were at 480 mμ and the remaining one, that of *Rht. glutinis* var. *aurantiaca* at 470 mμ abnormally. The absorption curves with a maximum at 450 mμ agreed fairly with those of the yellow *Rhodotorulas* though the color of cells were not similar to the latter.

When the above six cultures were incubated in potato-yeast medium, the absorption maxima of the petroleum ether extracts changed to 480 mμ without exception, and their curves agreed with those of the normal cultures of the red *Rhodotorula*. (Fig. 1) Indeed, the maximum at 450 mμ seems to have been obtained in a depressed condition of cells in which the yellow pigments predominate in the total carotenoids.

But in the yellow *Rhodotorulas*, the change of 450 mμ to 480 mμ could not be observed in the potato-yeast medium.

Formerly HASEGAWA (*7*) pointed out that the color of colony *Rhodotorulas* except *Rht. glutinis* var. *aurantiaca* and yellow *Rhodotorula* (*Rht. flava*) was distinctly limited in the range from yellowish red to orange. This specialization of the colony appearance accords with the above results. As both red and yellow pigments are included in the normal carotenogenesis of the red *Rhodotorula*, it is quite possible that, according to cultural conditions, the same absorption spectra of the total pigments as those of the yellow *Rhodotorula* are obtained in the former, even in the case that these two groups of *Rhodotorula* may be distinguishable each other taxonomically.

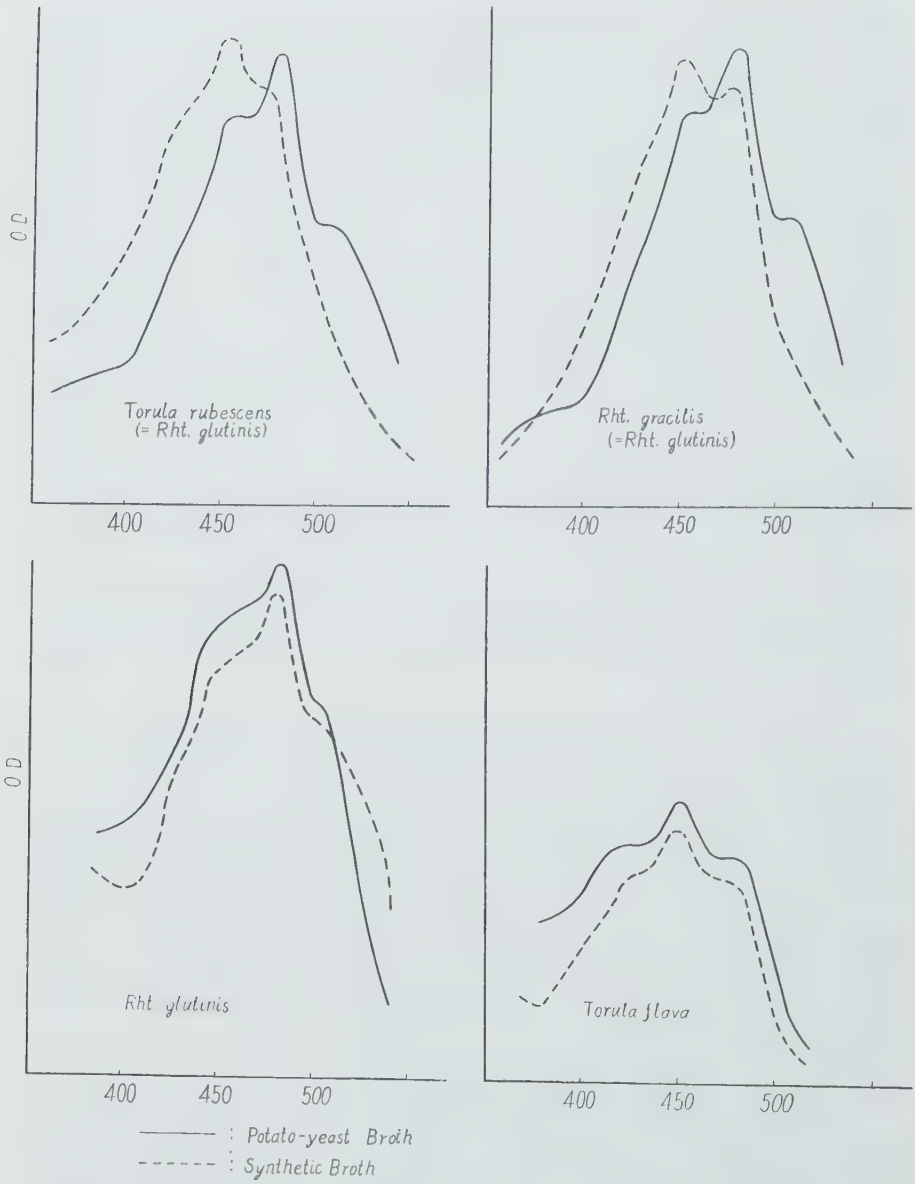

Fig. 1. The absorption curves of carotenoid pigments of *Rhodotorula* strains in two media.

Furthermore, from the test of the vitamin requirement on them, the difference between the two groups became still more clear.

In the red group, *Rhodotorula rubra*, *Rht. glutinis* var. *dairenensis*, *Rht.*

tokyoensis, *Rht. minuta* and *Rht. pallida* required thiamine as well as the yellow yeasts. In these cases, the requirement of thiamine by the red *Rhodotorulas* was stimulative and filled by the pyrimidine moiety only, while the yellow group essentially required both the moieties of thiamine; pyrimidine and thiazole. (Table 1) In the latter case, *Rht. flava* required both the moieties, but *Rht. peneaus* and *Rht. crocea* could be grown to a lesser extent by the supply of either moiety, which seemed to induce the synthesis of another one.

Table 1. Two types of the thiamine requirement by *Rhodotorula*

	Thiamine	P+T	P	T	None
Red Rhodotorula					
Rhodotorula rubra	100	100	100	18	14
Rht. glutinis var. *dairenensis*	100	70	70	30	30
Rht. tokyoensis (*Rht. marina* strain *tokyoensis*)	100	82	80	18	15
Rht. minuta	100	55	55	15	15
Rht. pallida	100	50	43	8	10
Yellow Rhodotorula:					
Rht. flava	100	100	12	5	5.5
Rht. peneaus	100	70	50	62	3
Rht. crocea	100	77	55	21	2.7

P: Pyrimidine moiety T: Tiazole moiety
Each value of the growth is shown in percentage of the optical density of a weak culture to that in the medium to which vitamins are completely added.

From these facts, the two colored groups in *Rhodotorula* should de differentiated. Therefore, we propose to name them the subgenera *Rubrotorula* nov. subgen. for red *Rhodotorulas*, and *Flavotorula* nov. subgen. for the yellow *Rhodotorulas*.

2. The general aspects of the subgenus *Rubrotorula*

The subgenus is characterized by the colony color due to the carotenoid pigment, which is limited strictly in the range between red and orange, and by the negative iodine reaction of the extracellular polysaccharide with the exception of *Rht. macerans* and *Rht. glutinis* var. *infirmominiata*.

The general aspects of the subgenus are described as follows.

1) Survey of the taxonomic members accepted in the subgenus

The following species and varieties are included.

Rhodotorula glutinis (Fres.) Harrison emend. Hasegawa
Rhodotorula glutinis (Fres.) Harrison var. *aurantiaca* (Saito) Hasegawa
Rhodotorula glutinis (Fres.) Harrison var. *dairenensis* Hasegawa et Banno

Rhodotorula rubra (DEMME) LODDER emend. HASEGAWA
Rhodotorula macerans SONNE FREDERIKSEN
Rhodotorula lactosa HASEGAWA
Rhodotorula marina PHAFF, MRAK et WILLIAMS
Rhodotorula texensis PHAFF, MRAK et WILLIAMS
Rhodotorula tokyoensis KOBAYASHI
Rhodotorula minuta (SAITO) HARRISON
Rhodotorula pallida LODDER

Among these, *Rhodotorula marina* and *Rht. tokyoensis* closely resembled each other. It was rather difficult to distinguish them in morphological and physiological properties except that of the stimulative requirement of thiamine by the latter. *Rht. tokyoensis* should be regarded as a strain of *Rht. marina*. In addition to these members, LODDER et al. (*16*) informed of *Rht. glutinis* var. *infirmo-miniata* as a starch former, the culture of which has not been yet examined by us.

2) The morphological property

In 1941, HENRICI (*27*) suggested in his paper that the size and shape of the cells of *Rhodotorula* were as highly variable as the shade of pigment. LODDER and KREGER-VAN RIJ touched upon the fact that although in some cases, both shape and size of the cells of yeast were often liable to considerable variations, the shape of cells could be regarded as a valuable characteristic for the differentiation of the species in *Rhodotorula*. HASEGAWA (*7*) pointed out that the taxonomic key of the *Rhodotorula* by LODDER and KREGER-VAN RIJ was misleading in the cell morphology. He examined 46 cultures which were lineal descendants of 12 old species published by SAITO and OKUNUKI, and often found remarkable differences in the shape of cells among descendants of the same strain. Moreover, this dimorphism afforded a sound explanation for the discrepancy between the descriptions by LODDER and KREGER-VAN RIJ and those by the original authors. From the results concerning the living organisms, besides the comparison between the descriptions by the original authors, by LODDER (1934) (4) and by LODDER, KREGER-VAN RIJ (1952) (*5*), the following conclusion could be derived.

Generally speaking, the round or short oval shape of cells seems to be fairly stable during the successive cultivation over a long period of time.

The oval cells are comparatively stable, but they often vary to long oval and elongate types during the successive cultivations. In a few cases, the oval cells changed to stable round and short oval type. The long oval and elongate cells are highly variable even with the change of cultural conditions. These phenomena seem to be a natural variation concerned with the activity of the cell division.

3) Assimilation of nitrate and carbon compounds

Among the physiological properties adopted into the taxonomy of yeasts, the assimilation of nitrate was confirmed to be much useful.

Many genera were distinguished by the ability to assimilate potassium

nitrate. But in the genus *Rhodotorula*, there were both nitrate positive and negative assimilabilities and the tests gave clear-cut results (*5*) (*12*).

Hasegawa and Banno (*12*) found within Saito's strains a red yeast which assimilated nitrate weakly, and named it *Rhodotorula glutinis* var. *dairenensis*. This weak assimilability has been kept in the organism unchanged since 1922. The fact was ascertained on the five descendants maintained in separate organs in Japan.

As to the carbohydrate assimilation, the 22 compounds were tested on the 27 cultures belonging to the subgenus (*6*). The results were as follows. Any remarkable difference between the tested organisms could not be observed in the assimilation of carbon compounds except three sugars used by Lodder and Kreger-van Rij.

glucose (+), galactose (+), maltose (+ or −), sucrose (+ or −) lactose (+ or −), rhamnose (−), raffinose (+), sorbitol (+) sorbose (+), mannitol (+), mannose (+), arabinose (+), xylose (+) dulcitol (−), inositol (−), adonitol (+), Na-succinate (+), salicin (+), K-2-ketogluconate (+), trehalose (+), α-methyl glucoside (+)

Skinner and Huxley (*28*) also examined 26 compounds on 78 *Rhodotorula* strains which were isolated by them and came to the same conclusion independently.

As to the ethanol assimilation, Lodder and Kreger-van Rij (*5*) stated that this property had not been used for the differentiation of species, and that it had only the secondary value in classification. According to our investigation, it also seems that the assimilability has not always been stable. Splitting of arbutin by all the members of the subgenus yielded a positive result.

4) Production of starch-like substance

Slime substances of capsulated yeasts were investigated by Mager (*29*), Mager and Aschner (*30*) Hehre *et al* (*31*) and Einbinder *et al* (*32*) Mager isolated from the cultures of *Torulopsis rotundata* and *Torulopsis neoformans* a carbohydrate material which stained blue with iodine, and described that the material was a mixture of starch and pentosan derived from the capsule of cells. Hehre *et al* obtained in crystalline form an amylose-like substance from the culture broth of *Torula hystolytica*. Mager and Aschner pointed out that the ability to produce the extracellular starch was restricted to the capsulated nonfermenting asporogenous yeasts. Lodder and Kreger-van Rij (5) used this property as one of the characteristics defining the genus *Cryptococcus*, admitting the fact that it could also be observed in several strains of *Bullera*, *Rhodotorula*, *Candida* and *Trichosporon*.

Rht. peneaus (*8*) in *Flavotorula*, and *Rht. macerans* (*11*), *Rht. glutinis* var. *infirmo-miniata* (*16*) in *Rubrotorula* were reported as the starch producing *Rhodotorula*.

In our experiment, the shaking culture broths of *Rht. peneaus* and *Rht. macerans* also showed a clear iodine reaction. Recently, it was found (*16*) that, unlike Cryptococcus cultures, all the strains of *Rhodotorula* that res-

ponsed positively to the starch reaction could develop the substances without cultivation on a medium at low pH.

5) Phylogenetic relationship of the species

Generally speaking, in the field of asporogenous yeasts, it is difficult to consider as to the phylogenetic relationship between the species, because each species is based on the differentiation of too much simplified characters. But in the genus *Rhodotorula*, there was found a very interesting fact that the nature of the vitamin requirement among the strains were related to the species or groups of species based on the other taxonomic properties. Therefore, with the help of several serological data of BENHAM (*33*) SEELIGER (*34*) and TSUCHIYA *et al* (*35*) a phylogenetic consideration was examined on the species of the subgenus.

SKINNER and HUXLEY (*28*) were inclined to belive that the genus *Rhodotorula* should be considered monotypic. As one of the reasons for this, the independency of *Rht. glutinis* in the nutrition of vitamins was given against the other *Rhodotorula* species. This presumption, however, is hardly to be admitted on the ground of the serological studies mentioned above. But, from the genealogical point of view, the value of their opinion should be appreciated. We also recognize *Rht. glutinis* as the ancestral organism on account of more predominant distribution in the nature than the other members of the genus as well as its autotrophic tendency on the vitamin nutrition.

The genus *Rhodotorula* has, in the main, three phylogenetic groups. Among these, two groups are included in the subgenus *Rubrotorula* as shown in Figure 2, and the remaining one belongs to the subgenus *Flavotorula*. The first group in *Rubrotorula* is indicated as a phylogenetic line including *Rht. glutinis* and *Rht. rubra* (*36*). The members of the group showed the least requirement of vitamin in the genus. Though the strains belonging to *Rht. glutinis* tested by us showed no vitamin requirement, *Rht. glutinis* var. *dairenensis* and *Rht. rubra* required thiamine stimulatively, with the exception of one strain. (Table 2) The exceptional strain " ? *Torula sanguinea*" (=*Rht. rubra*, SAITO 1922) required vitamins variably in the four descendants. Two descendants required niacin essentially and another one required inositol stimulatively and the remainder, as well as *Rht. glutinis* var. *aurantiaca*, responsed to the mixed vitamins. The plurality in the vitamin requirement indicates that the original culture of this strain was in a variable condition when it was isolated by the author.

On the other hand, *Rht. glutinis*, *Rht. glutinis* var. *dairenensis* and *Rht. rubra* form a degenerating line on the nitrate utilizability. SEELIGER (34) separated *Rht. glutinis* from *Rht. rubra* and *Rht. mucilaginosa* (=*Rht. rubra*) through serological precipitation reactions. TSUCHIYA *et al.* (35) reported the results of the thermostable antigen analysis of six species of *Rhodotorula*. In the work, they proved three common antigens No. 1, No. 2, No. 5 between *Rht. glutinis* and *Rht. glutinis* var. *aurantiaca*. Two of them, the antigens No. 1, No. 2, and a different antigen No. 3 were also confirmed in *Rht. rubra*,

Table 2. The requirement of vitamins by *Rubrotorula*

Species	Strain names	Cultures No.	B_1HCl	PABA	Biotin	Niacin	Pantothenate	Inositol	B_6HCl	B_2
Rhodotorula rubra	*Cryptoc. ruber*	NCYC 68	S	—	—	—	—	—	—	—
	Cryptoc. rubrorugosus	IFO, 0004	S	—	—	—	—	—	—	—
	〃	NCYC 64	S	—	—	—	—	—	—	—
	〃	NRRL Y-843	(E)							
	Cryptoc. pararoseus	IFO, 0003	S			—	—	—	—	
	〃	NRRL Y-844	S	—	—	—	—		—	
	Blastodendrion carbonei	IFO, 0001	S		—	-	—	—	—	
	Blastodendrion simplex	IFO, 0002	S		-	-	—	—	—	
	Torula mucilaginosa	NCYC 63	S	—	—	—	—	—	-	
	〃	NRRL Y-168	S		-		—			
	Torula sanguinea	NCYC 65	S				-			
	〃	NCYC 66	(E)	-				—	—	
	〃	NRRL Y-174	S	—	—	—	—	—	-	
	Torula corallina	IFO, 0406	S	—						
	Torula decolans	IFO, 0383	S							
	Torula sanguinea?	IFO, 0712	—	—	—	E	—	—	—	—
	〃	Cul, 18	—	—	—	E	—	—	—	—
	〃	Cul, 19	—	—	—	—	—	S		
Rhodotorula minuta		IFO, 0387	S	E			—	—		
〃		Cul, 20	S	E			—	—	—	
Rhodotorula pallida		CBS, L-320	(E)	E	-		-	—	—	—
Rhodotorula glutinis var. *dairenensis*		IFO, 0415	S	—	—	-	—	—	-	
〃		Cul, 14	S	—	—	—	—	—	—	—
Rhodotorula lactosa		IFO, 1006	—	E	—	-	—	-	—	—
Rhodotorula macerans		CBS, 2425	—	—	E	—	—	—	—	—
Rhodotorula marina		CBS, 2365	—	E	—	—	—	—	—	
Rhodotorula marina strain *tokyoensis*		IFO, 0879	S	E	—	—	—	—	—	—
Rhodotorula texensis		CBS, 2177	—	E	-	-	—		—	—

S; Stimulative requirement,
E; Essential requirement, (E); Doubtful essential requirement
NCYC; National Collection of Yeast Cultures, England
NRRL: Northern Utilization Research Branch, USA
CBS; Centraalbureau voor Schimmelcultures, Holland
IFO; Institute for Fermentation
Cul.; Other lineal descendant from the origin of the above IFO culture

although the antigen No. 2 could not be found in *Rht. mucilaginosa*.

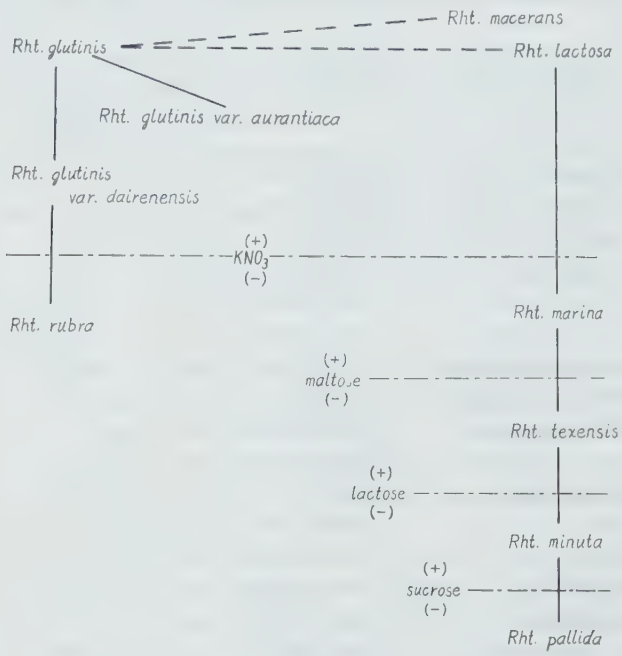

Fig. 2. The phylogenetic relationship of *Rubrotorula*.

The second group is shown as another line consisted of *Rht. lactosa*, *Rht. marina*, *Rht. texensis*, *Rht. minuta* and *Rht. pallida*. The characteristics of this group are the assimilability of lactose with the exception of *Rht. pallida*, and an essential requirement of para-amino-benzoic acid (*36*) (*37*). (Table 2). A stimulative requirement of thiamine was also observed in the group; i.e. *Rht. marina* strain *tokyoensis*, *Rht. minuta* and *Rht. pallida*. The thiamine requirement in the two groups of *Rubrotorula* was filled with the pyrimidine moiety, and in this point, the subgenus is distinguishable from *Flavotorula* (*38*). (Table 1)

From another point of view, this line indicates a degenerating tendency on the assimilabilities of nitrate and sugars, i.e. maltose, lactose, and sucrose. SKINNER and HUXLEY (*28*) observed a weak assimilation of lactose in *Rht. minuta*, using the authentic strain from CBS and five strains of 27 isolates belonging to the species, although the species had been regarded as a lactose non-assimilating organism. Actually, *Rht. minuta* closely resembled *Rhodotorula texensis* in the taxonomic properties except the assimilability of lactose and the requirement of thiamine. Generally speaking, the assimilability of lactose in this group has somewhat a variable inclination. So, it is quite possible that *Rht. minuta* is closely related to *Rht. texensis*. The right taxonomic position of *Rht. minuta* is considered to be a variety of

Rht. texensis.

Benham (*33*) and Seeliger (*34*) separated *Rht. minuta* and *Rht. pallida* from other *Rhodotorula* species serologically. As to the two species, Tsuchiya *et al* proved a common antigen and another one in each of them; i.e., antigen No. 4 and No. 7 in *Rht. minuta*, and No. 4 and No. 6 in *Rht. pallida*, but No. 1, No. 2 and No. 3 could not be found in both species. The thermostable antigens of *Rht. marina* strain *tokyoensis* were designated by Yonezawa (*39*) as No. 4 and No. 7 which were common with *Rht. minuta.*

There has been found no ascertained relationship between *Rht. macerans* and the above two groups. In *Rubrotorula*, *Rht. macerans* was characterized by the starch formation and by the essential requirement of biotin instead of para-aminobenzoic acid and of thiamine. As to the starch formation, the existence of *Rht. glutinis* var. *infirmominiata* should be remembered. (This strain was regarded by Lodder and Kreger-van Rij as a synonym of *Rht. glutinis.*) From this, *Rht. macerans* as well as *Rht. lactosa* is supposed to have some phylogenetic relationships to *Rht. glutinis.*

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EXPLANATION OF PLATE VI

The color shades of the Rhodotorula cell (I)

The colored cells were obtained by shaking culture with the following media: a) Synthetic medium, b) Peptone medium, c) Potato-yeast-extract medium

1) Rhodotorula glutinis (FRES.) HARRISON (NCYC, 59; HARRISON's strain)
2) Rhodotorula gracilis RENNERFELT (=Rhodotorula glutinis)
3) ? Torula rubra SCHIMON=Torulopsis saitoi Cif. et Red. (=Rhodotorula glutinis)
4) Torula koishikawensis OKUNUKI (=Rhodotorula glutinis)
5) Torula rubescens SAITO (=Rhodotorula glutinis)
6) Torula mucilaginosa JÖRGENSEN (=Rhodotorula rubra (DEMME) LODDER)
7) Torula minuta SAITO (=Rhodotorula texensis PHAFF, MRAK et WILLIAMS var. minuta (SAITO) nov. var.)
8) Torula rubra var. α SAITO (=Rhodotorula glutinis var. dairenensis HASEGAWA et BANNO)
9) Torula aurantiaca SAITO (=Rhodotorula glutinis var. aurantiaca (SAITO) HASEGAWA)
10) Torula flava SAITO (=Rhodotorula flava (SAITO) LODDER)
11) Rhodotorula crocea SHIFRINE et PHAFF
12) Rhodotorula peneaus PHAFF, MRAK et WILLIAMS

1a 1b 1c 2a 2b 2c 3a 3b 3c

4a 4b 4c 5a 5b 5c 6a 6b 6c

7a 7b 7c 8a 8b 8c 9a 9b 9c

10a 10b 10c 11a 11b 11c 12a 12b 12c

The color shades of the Rhodotorula cell.

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"AN ANALYSIS ON BUBBLING OF BACTERIA-BORNE AIR"

SHUICHI AIBA, TOYOKAZU YAMADA and KAETSU KOBAYASHI

The Institute of Applied Microbiology, University of Tokyo

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INTRODUCTION

"Bubble aeration" technique is extensively used in laboratory- and industrial-scale fermentations for both oxygen transfer and agitation. It goes without saying that the air for this operation must be sterilized. Fibrous filters are routinely used for the sterilization. However, it seems interesting to study how the infectious bacteria or other microbes which should happen to pass through the air filters uncollected into the culture media are collected by the latter. If the collection efficiency is high enough, a procedure to supplement the air filter performances might be developed. If not, the information on the transfer of microbes from air bubbles into surrounding liquids might be of help in securing a basis for the design and operation of the air sterilization filters.

It is the purpose of this paper to make an experimental approach to the transfer of microbes in using the "bubble aeration" technique.

TRANSFER MECHANISM

In considering the mechanisms of transfer of microbes from air bubbles into liquids, the following assumptions were made.

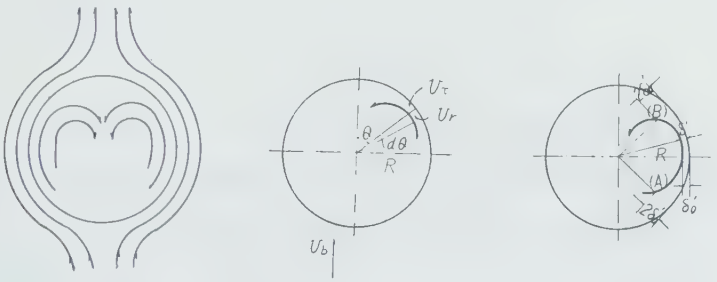

Fig. 1. Flow pattern of air inside bubble.

1) Air bubbles are spherical.
2) Spherical microbes are uniformly distributed inside bubbles.
3) Within each bubble air is circulated. The flow pattern is assumed

to be that which is shown in Fig. 1a and the tangential velocity of circulation is also assumed as follows (1):

$$v_\tau=(1.5)\cdot v_b\cdot\sin\theta \qquad \text{(see Fig. 1b)}$$

4) The flow is laminar and the mechanisms of transfer based on the centrifugal force and the Brownian motion are considered exclusively.

5) Microbes are collected by surrounding liquids once they collide with liquid-air interfaces.

a) Transfer by the centrifugal force

The centrifugal settling velocity of microbial particles is derived from Stokes' law as shown in Equation (1).

$$v_r=\frac{\delta^2(\rho-\rho_0)}{18\mu}\cdot\frac{v_\tau^2}{R}=(2.25)\cdot\frac{\delta^2(\rho-\rho_0)}{18\mu}\cdot\frac{v_b^2}{R}\cdot\sin^2\theta \qquad (1)$$

The number of particles collected by liquids during the period of time Θ,

$$\begin{aligned} n' &= \int_0^\pi v_r\cdot(2\pi R\sin\theta)\cdot R\cdot d\theta\cdot n\cdot\Theta \\ &= \frac{(2.25)\cdot\delta^2\cdot(\rho-\rho_0)}{18\mu R v_b}\cdot 2\pi R^2\cdot n\cdot H\cdot\int_0^\pi \sin^3\theta d\theta \\ &= \frac{\delta^2(\rho-\rho_0)}{3\mu}\cdot v_b\cdot\pi R\cdot n\cdot H \end{aligned} \qquad (2)$$

On the other hand,

$$n_0=\left(\frac{4}{3}\right)\cdot\pi R^3 n \qquad (3)$$

Then,

$$\eta_1=\frac{n'}{n_0}=\frac{\delta^2(\rho-\rho_0)}{4\mu R^2}\cdot v_b\cdot H \qquad (4)$$

b) Transfer by the Brownian motion (1)

An infinitesimal volumetric flow rate of circulation is assumed as follows:

$$\Delta V=2\pi R\cdot\sin\theta\cdot\delta'\cdot v_\tau=3\pi R\cdot v_b\cdot\sin^2\theta\cdot\delta'=3\pi R\cdot v_b\cdot\delta_0' \qquad (5)$$

(see Fig. 1c)

The space extending from $\theta=(\pi/4)$ to $\theta=(3/4)\pi$ is conveniently taken into account for computing the rate of transfer due to diffusion.

From Equation (5),

$$\left.\begin{aligned} v_b\cdot\left(\frac{1}{2}\right)\cdot\delta' &= v_b\cdot\delta_0' \\ \delta' &= 2\delta_0' \end{aligned}\right\} \quad \left(\theta=\frac{\pi}{4},\ \frac{3}{4}\pi\right) \qquad (6)$$

The time Θ' required for microbial particles to travel from (A) to (B),

$$\Theta'=\int_{\pi/4}^{(3/4)\pi}\frac{Rd\theta}{v_\tau}=\frac{R}{(1.5)v_b}\int_{\pi/4}^{(3/4)\pi}\frac{d\theta}{\sin\theta}=(1.17)\left(\frac{R}{v_b}\right) \tag{7}$$

The mean radial distance,

$$\bar{\delta}'=(1.5)\cdot\delta_0' \tag{8}$$

The radial distance δ'' of transfer during the time Θ' will be,

$$\delta''=2\sqrt{\frac{D\Theta'}{\pi}}=2\sqrt{\frac{(1.17)\cdot DR}{\pi v_b}}=(1.22)\cdot\sqrt{\frac{DR}{v_b}} \tag{9}$$

The number of particles collected per unit time is assumed to be that which is containd in the air volume defined by,

$$3\pi Rv_b\cdot\delta_0' \quad \begin{pmatrix}\delta_0'=\frac{2}{3}\cdot\bar{\delta}' \\ \bar{\delta}'-\delta''\end{pmatrix} \tag{10}$$

Then,

$$n'=3\pi Rv_b\cdot\delta_0'\cdot n\cdot\Theta=3\pi R\cdot v_b\cdot\left(\frac{2}{3}\right)\cdot(1.22)\sqrt{\frac{DR}{v_b}}\cdot n\cdot\Theta$$
$$=(2.44)\cdot\pi R\cdot\sqrt{\frac{DR}{v_b}}\cdot n\cdot H \tag{11}$$

$$\eta_2=\frac{n'}{n_0}=(1.83)\cdot\sqrt{\frac{D}{v_b\cdot R^3}}\cdot H \tag{12}$$

The over-all collection efficiency η is assumed as follows:

$$\eta=\eta_1+\eta_2 \tag{13}$$

EXPERIMENTAL APPARATUS AND PROCEDURE

The flow diagram of this series of experiments is shown in Fig. 2a. Bacteria used were *Serratia marcescens*. The culture media contained glucose, peptone, KH_2PO_4 and $MgSO_4\cdot 7H_2O$, amounting to 15, 0.5, 0.5 and 0.2% respectively (pH=6.8–7.0). After 24 hrs., at 30°, the concentrated cells were diluted by about one tenth using sterilized water (pH=7.0).

Air from a compressor, after passing through air chambers and a dryer of calcium chloride, was used to atomize a certain amount (15 ml) of liquid suspension of the bacterial cells, the preparation of which was repeated prior to each experiment as described before.

An aquarium made by cementing four glass plates and rectangular in shape was used throughout these experiments (Fig. 2b). At its bottom, a

nozzle of brass was fixed. Through this nozzle, the bacteria-borne air was blown into water which was filled to a certain depth (9–12 cm) of the aquarium in each experiment. The sterilized water (pH=7.0) was used as liquid.

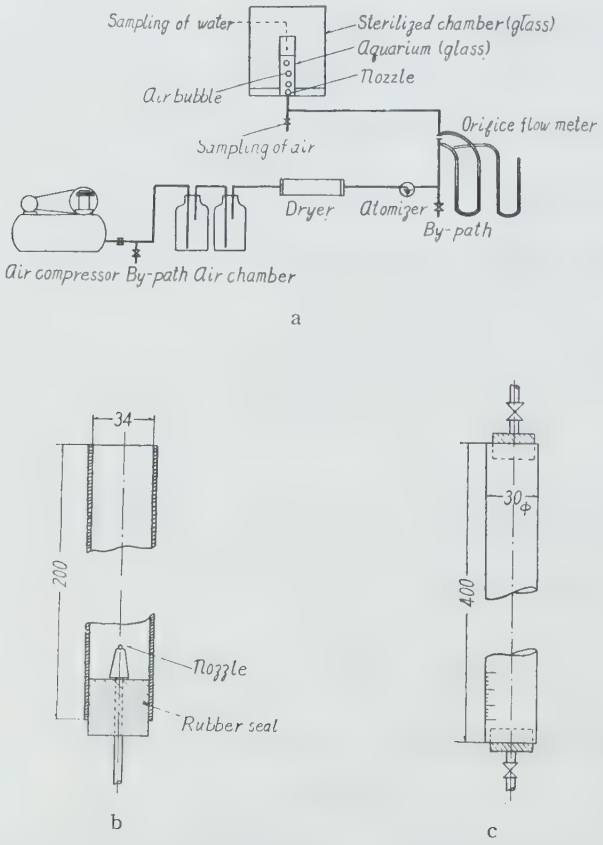

Fig. 2. Flow diagram and experimental apparatus used.

Four nozzles, different in diameter covering 0.2, 0.7, 1.0 and 1.8 mm, were used to obtain air bubbles of different sizes. In each case, air flow rate through the nozzle was adjusted so that single or "chain-like" bubbles (2) could be formed. It is because of the fact that air bubbles' deformations or ruptures caused by the turbulent air-liquid flow were prohibitive for analysing experimental data.

Sampling of liquid was made several times in a certain interval during each experiment. Namely, a definite amount of liquid, 1 ml in volume, was taken by a graduated pipett in this sampling. The liquid was distributed to three Petri dishes by 0.1 ml each. Then, about 10 ml of the culture medium (peptone 1.0%, meat extract 1.0%, NaCl 0.3% and agar-agar 2%, pH=6.8–7.0)

was added into each dish. After 24 hrs., at 33°–36°, the colonies were counted. In each sampling, such a procedure was repeated.

It was important to obtain the most reliable data concerning the number concentration of the bacterial cells in the air which was blown into the liquid. After several trials, the procedure of air sampling which was considered dependable was as follows:

Under each condition of bubbling, a glass cylinder (Fig. 2c), completely filled with sterilized water was displaced by the bacteria-borne air (about 240 ml in each case). The displacement was made under exactly the same reading of an orifice meter as that which was recorded before this operation. Next, the residual liquid (about 45 ml) in the sampling cylinder was thoroughly mixed with the introduced air by manual shaking for about 2 min. Then, the liquid was pipetted and distributed equally by 0.1 ml into three Petri dishes. The procedure of determining the viable concentration of the liquid was the same as that mentioned before. All these experimental procedures were made, free from infections, of course.

Photographs of bubbles relating to each experimental condition were taken to determine the volume-surface mean radii.

EXPERIMENTAL RESULTS AND DISCUSSIONS

Fig. 3 shows one of the experimental results. The ordinate is the viable number concentration of the cells n'' in the liquid, while the abscissa represents the duration of time Θ'' of this experiment. The scattering of data of plate counting is shown by broken lines around data points. The deviations of most of the data obtained throughout these experiments were of the order below those illustrated in Fig. 3. The linear relations between n'' and Θ'' were noted in each experimental result as shown in Fig. 3.

Experimental results of η which were obtained from these linear relations are summarized in Fig. 4, the ordinate and the abscissa of which being the efficiency per unit depth (cm) of the liquid and the bubble diameter, respectively. Although these data points are scattered rather widely, the tendency that η is gradually decreased contrary to the increase of R is noted. The reason for the scattering of data points is complicated. The unexpected error in the determination of n_0 might account for the scattering. It was ascertained that n_0, obtained from the other procedure than that described before, for instance, by impinging the bacteria-borne air into the liquid at standstill, resulted in a further deviation of η.

The over-all collection efficiency η per unit depth of liquid which was calculated from Equations (4) and (12) is also included in Fig. 4, the parameter being the ascending velocity v_b of bubbles. In this calculation, the diffusion coefficient of *Serratia marcescens* in air bubbles was estimated, $D=2.36\times10^{-7}$(cm²/sec), assuming $\delta = 1.0 \times 10^{-4}$(cm) and $\mu = 1.81 \times 10^{-4}$ (gr/cm·sec). (Einstein's formula). The viscosity and density (μ, ρ_0) of air were assumed as those corresponding to 20°C. Furthermore, the density of the

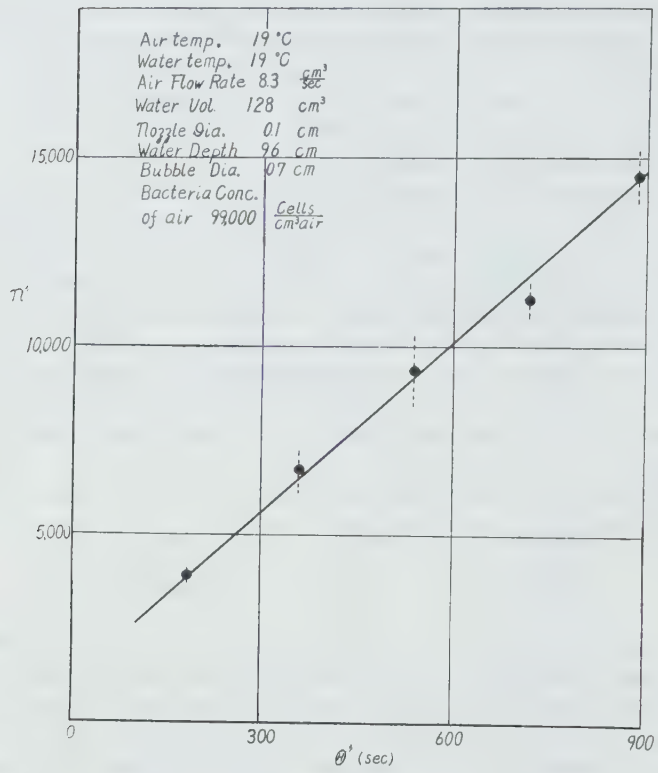

Fig. 3. Linear relation between n'' and Θ''

bacterial cell was assumed, $\rho=1.0(\mathrm{gr./cm^3})$.

It is seen from this calculation that η_1 due to the centrifugal force is predominant. The tendency that η decreases conversely with the increase of R agrees with that of experimental results. The range of the ascending velocity v_b of bubbles of these experiments was estimated to be around 20–30 cm/sec. Therefore, the quantitative agreement between the experimental data and the results of calculation is not satisfactory. The reasons of this disagreement remain to be studied. However, the assumptions which were too simplified to describe precisely the transient situation of bubble formation and its vibrating motion during the ascent through the liquid might have been its principal cause of the discrepancy.

If the culture media, the physical properties being different from those of water, were used as liquids, bubble sizes might have been affected. In the case of small bubbles and deep liquid submergence, a high efficiency of collection of microbes is expected from Fig. 4.

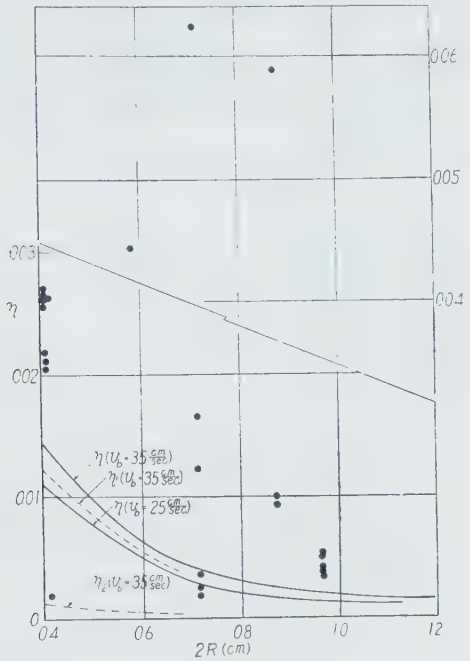

Fig. 4. Correlation between over-all collection efficiency and bubble diameter.

SUMMARY

The transfer of microbes from air bubbles into liquids was analysed using the bacterial cells of *Serratia marcescens*.

Experimental data were compared with the calculations which were derived from several assumptions. The agreement between them was not satisfactory. The reasons of this disagreement remain to be investigated.

The "bubble aeration" technique to supplement the air filter performances seems promising so far as the collection of microbes in the case of small bubbles and deep tanks are solely concerned.

NOMENCLATURE

D	=diffusion coefficient of bacterial cell	(cm^2/sec)
H	=liquid depth	(cm)
n	=number concentration of bacterial cells in air bubble	($1/cm^3$)
n'	=number of bacterial cells removed during the time Θ	
n''	=number concentration of bacterial cells in liquid	($1/cm^3$)
n_0	=initial number of bacterial cells in air bubble	
R	=bubble radius	(cm).

v_b	=ascending velocity of air bubble	(cm/sec)
v_r	=centrifugal settling velocity of bacterial cell	(cm/sec)
v_τ	=tangential velocity of air inside bubble	(cm/sec)
ΔV	=infinitesimal volumetric flow rate of air inside bubble	(cm^3/sec)
δ	=diameter of bacterial cell	(cm)
δ'	=infinitesimal radial distance from bubble surface	(cm)
δ_0'	$=\delta'$ at the equator of air bubble	(cm)
$\bar{\delta}'$	=mean of δ'	(cm)
η	=over-all collection efficiency	
η_1, η_2	=collection efficiencies based on the centrifugal force and the Brownian motion, respectively	
μ	=viscosity of air	(gr/cm-sec)
ρ	=density of bacterial cell	(gr/cm^3)
ρ_0	=density of air	(gr/cm^3)
θ	=azimuth	
Θ	=holding time of air bubble in liquid	(sec)
Θ'	=time required for bacterial cells to move a certain distance	(sec)
Θ''	=duration of time of air bubbling	(sec)

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FURTHER NOTES ON THE NATURE OF SULFUR-CONTAINING PEPTIDE-NUCLEOTIDE COMPLEX OBTAINED FROM YEAST

EIJI HASE and SAYOKO MIHARA

The Institute of Applied Microbiology, University of Tokyo, and the Tokugawa Institute for Biological Research, Tokyo

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In our previous papers (*1*, *2*, *3*) it has been reported that a sulfur-containing peptide-nucleotide complex is present in the cold trichloroacetic acid (TCA)-soluble fraction of *Chlorella* and yeast cells, and that the complex seems to play some important role in the cell division of *Chlorella* (*4*, *5*). The electrophoregrams of the complexes isolated from *Chlorella* and *Saccharomyces formosensis* obtained at acidic pH levels have already been presented in our preceding paper (*2*). The complex isolated from *S. formosensis* moved toward the cathode forming ostensibly a single peak at pH levels of 3.6–5.0, while the complex obtained from *Chlorella* split into several components at pH 5.0 and most of the components moved toward the anode. The present paper describes further electrophoretic data obtained at pH levels of 7.0 and 9.5 with the complex compound in question isolated from *S. formosensis*. Some data dealing with the modification of the complex caused by urea and formamide, which have been known to split the hydrogen bonds in complex molecules (*6*), will also be reported.

The cold TCA-extract of yeast cells was prepared by the same procedures as before. When the pH of the sample obtained was adjusted to 7.0 or 9.5, a considerable amount of precipitates was produced. Ethylenediamine tetraacetic acid was found to be able to bring most of the precipitates into solution. The insoluble materials yet remaining were centrifuged, and the clear solution obtained was applied to zone electrophoresis using starch as the medium. The procedures and experimental conditions employed in the zone electrophoresis were, unless otherwise designated, the same as those used in our preceding study (*2*). As can be seen in Figs. 1 and 2, almost all of the sulfur substances moved toward the cathode even at these neutral and alkaline pH levels together with the UV-absorbing substances forming a single peak. This result gives further support to our previous assumption that certain strongly basic substance(s) may be bound to the complex, although its chemical nature remains unknown yet. Fig. 3 shows the ultraviolet absorption spectra of the peak fraction (I in Fig. 1) isolated by the zone electrophoresis at pH 7.0. Similar absorption spectra were obtained also with the peak fraction separated by the zone electrophoresis at pH 9.5. The UV-

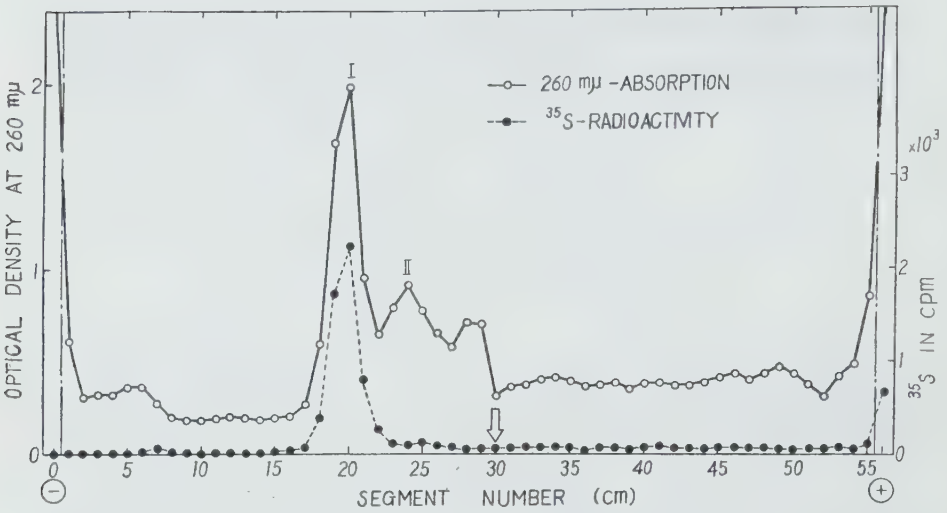

Fig. 1. Electrophoretic pattern of TCA-extract of *Saccharomyces formosensis* obtained on starch in phosphate buffer (0.02 M) of pH 7.0 after 39 hours at ca 3.5 v/cm. The arrow in the figure shows the place of application of the original sample. Segment numbers 0 and 56 represent the buffer reservoirs placed at both ends of the cell. The values given for these segments represent—with reference to the values given for the other segments—the total quantities of sulfur and 260 mμ-absorbing substances migrated to these reservoirs.

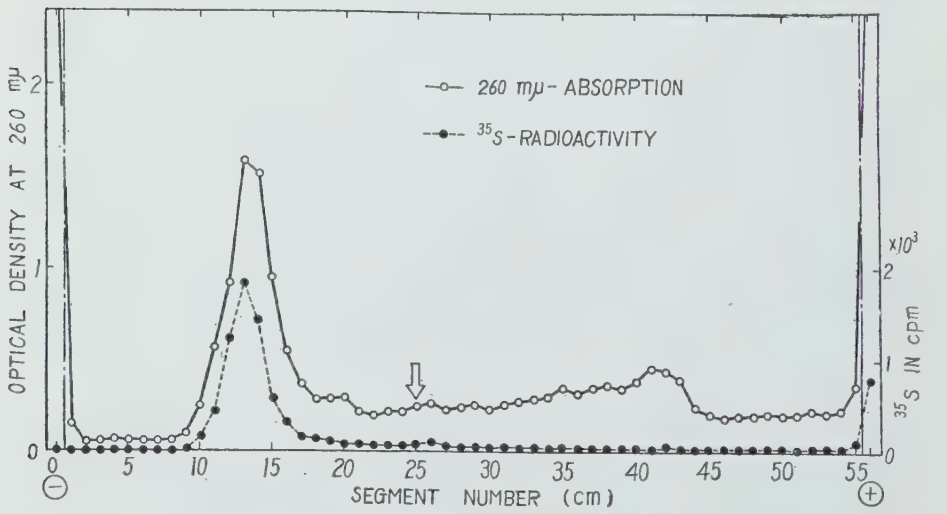

Fig. 2. Electrophoretic pattern of TCA-extract of *Saccharomyces formosensis* obtained on starch in triethylammonium carbonate buffer (7) of pH 9.5 (0.025 M) after 38 hours at ca. 4.5 v/cm. See also the legend of Fig. 1.

absorbing substance in the fraction designated as II in Fig. 1, seemed to be a hypoxanthine derivative, as judged from its characteristic absorption spectra having maxima at 250 mμ at the neutral or acidic pH levels. Paperchromatography of the sulfur substances isolated by these electrophoretic procedures revealed that they were the peptide containing cyst(e)ine as a major sulfur component which has already been described in our previous paper (*1*, *2*, *3*). These results indicate that almost all of the sulfur substances in the cold TCA-soluble fraction of the yeast cells studied form a peptide which is bound to the nucleotide moiety throughout the pH levels ranging from 3.6 to 9.5, although the nucleotide moiety seems to split to some extent at neutral or alkaline pH levels.

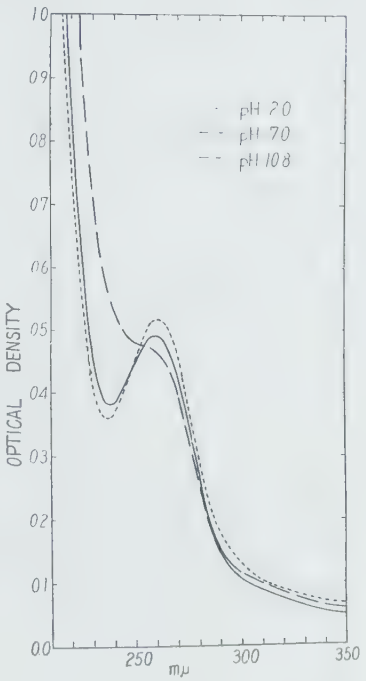

Fig. 3. Ultraviolet absorption spectra of the peak fraction I in Fig. 1.

It has been reported in our previous papers (*1*, *2*, *3*) that the components forming the complex isolated from *Chlorella* are bound together by rather weak forces which are readily affected by pH, ethanol-treatment, and anion-exchange chromatography. To obtain further insight into the nature of the forces binding the components in the case of yeast, the effects of urea and formamide upon the bondings involved were examined. Figs. 4 and 5 demonstrate the electrophoregrams obtained with the urea (8 M)- and formamide (10 M)-treated TCA-extracts, respectively. As can be seen from these figures, considerable changes were produced in the electrophoretic pattern, especially in the case of formamide-treatment. In the control run, the sulfur substances moved together with the UV-absorbing substances forming a single peak, whereas the sulfur substances treated with formamide were separated in a number of smaller fractions. Judging from the ultraviolet absorption spectra, the UV-absorbing substance located on segment No. 4 in the case of formamide-treated sample seemed to be identical with that found in the corresponding segment in the control (probably polynucleotide), while the UV-absorbing substance in segment No. 7 was found to be formamide, which was accompanied by the highest peak of sulfur substances. This result suggests that the sulfur substances originally bound to the nucleotide moiety of the complex were split off by the treatment with formamide and might have recombined with the formamide. At any rate, these observations lead us to a conclusion that the hydrogen bonds are partly involved in the struc-

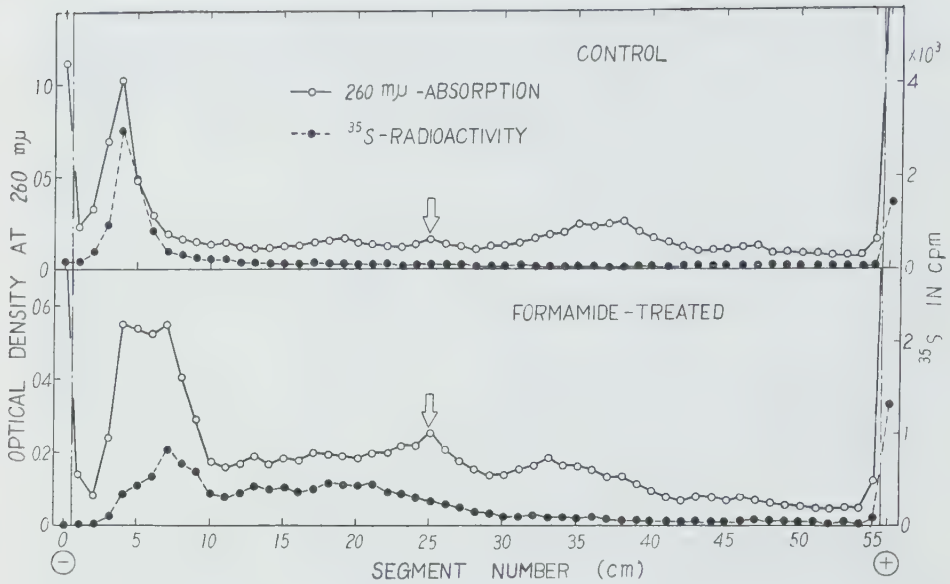

Fig. 4. Electrophoretic patterns of formamide-treated (lower) and non-treated (upper) TCA-extracts of *Saccharomyces formosensis* obtained on starch in triethylammonium carbonate buffer of pH 9.5 after 39 hours at ca. 5 v/cm. The cell used consisted of two troughs separated from each other with an acrylate plate, each trough having a dimension of $2 \times 1.5 \times 55$ cm. The TCA-extract had been in contact with formamide (10 M) for about one hour at pH 9.5. See also the legend of Fig. 1.

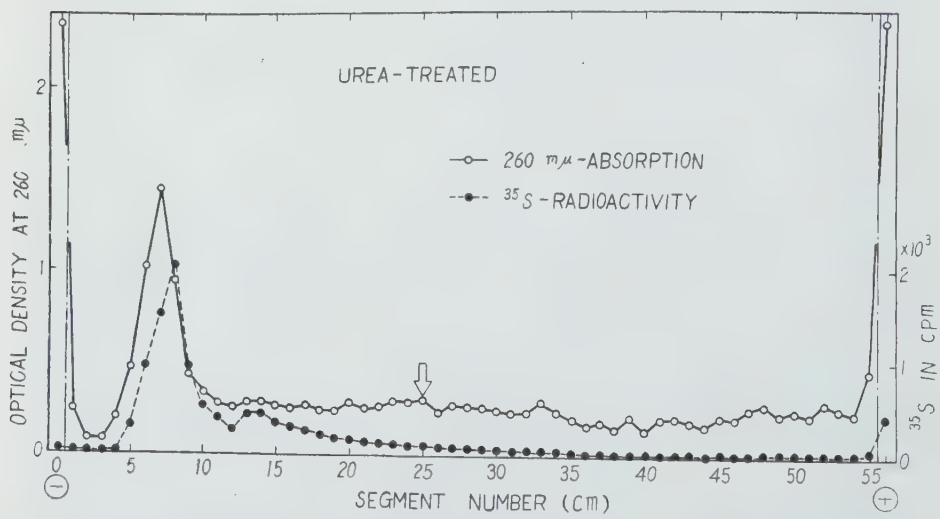

Fig. 5. Electrophoretic pattern of urea-treated TCA-extract of *Saccharomyces formosensis* obtained on starch in triethylammonium carbonate buffer of pH 9.5 after 39 hours at ca. 5 v/cm. The TCA-extract had been in contact with urea (8 M) for 3.5 hours. See also the legend of Fig. 1.

ture of the sulfur-containing peptide-nucleotide complex studied.

We wish to express our heartfelt thanks to Professor H. TAMIYA for his advice and encouragement in this work. We are also grateful to Dr. K. SHIBATA for his valuable suggestions, and to Dr. H. IIZUKA who kindly supplied the yeast strain to us. This work was largely supported by the grants from the Ministry of Education and the Rockefeller Foundation.

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Abstract

Reports of studies conducted by members of the Institute of Applied Microbiology, University of Tokyo, published elsewhere are abstracted.

J. Biochem., **46**, 521–524 (1959)

Formation of Phycobilin Pigments in a Blue-Green Alga, *Tolypothrix tenuis*, as Induced by Illumination with Colored Lights

By Akihiko HATTORI and Yoshihiko FUJITA

Division of Biosynthesis II

The chromatic adaptation in a blue-green alga, *Tolypothrix tenuis*, was investigated through spectroscopic analysis of the organism under illumination with various colored lights.

A marked increase in phycoerythrin content was observed on illuminating the organism with blue and green lights; no increase by red and pink lights. The production of phycocyanin and allophycocyanin, on the other hand, was found to be strongly enhanced by purple, red and pink lights but not by blue and green lights. Judging from the optical characteristics of the color filters used in these experiments, it was concluded that the spectral ranges of the lights effective to the formation of each phycobilin species lie at wave lengths from 550 to 700 mμ for phycocyanin and allophycocyanin and at those around 500 mμ for phycoerythrin.

J. Biochem., **46**, 633–644 (1959)

Crystalline Phycobilin Chromoproteids Obtained from a Blue-Green Alga, *Tolypothrix tenuis*

By Akihiko HATTORI and Yoshihiko FUJITA

Division of Biosynthesis II

From the cells of a fresh-water blue-green alga, *Tolypothrix tenuis*, the three components of phycobilin pigments (phycocyanin, phycoerythrin and allophycocyanin) were isolated and purified to crystalline states by combined use of salting-out method (using ammonium sulfate) and adsorption method (using tricalcium phosphate-gel) followed by the technique of ultracentrifugation.

Using the highly purified samples, determinations were made of the various physicochemical properties of the chromoproteids, including the sedimentation and diffusion constants, electrophoretic mobilities, isoelectric points, molecular weight and elementary composition, and the results obtained were compared with those reported earlier by other workers.

J. Biochem., **46**, 903–909 (1959)

Spectroscopic Studies on the Phycobilin Pigments Obtained from Blue-Green and Red Algae

By Akihiko HATTORI and Yoshihiko FUJITA

Division of Biosynthesis II

Three kinds of phycobilin pigments (phycocyanin, phycoerythrin and allophycocyanin) were isolated and purified from various algae including 4 species of Rhodophyceae and 4 species of Cyanophyceae. Spectroscopic data characterizing the purified pigments were presented, and compared with those reported by earlier investigators.

While phycocyanin and allophycocyanin obtained from different algal species were practically identical in their spectroscopic properties, the properties of phycoerythrins varied widely according to their sources. The difference was observed not only with algae of different classes, but also with the algae belonging to the same class (Rhodophyceae). The common spectroscopic

feature of phycoerythrins from different sources was the absorption bands at 565, 545, 500, 370, 310 and 280 mμ, the relative intensity of which varied, however, widely from sample to sample.

Allophycocyanin was found to be far widely distributed among blue-green and red algae than it has been thought heretofore. It was found to share with phycocyanin the property of showing absorptions or shoulders at 280, 285 and 360 mμ.

Relative content of the three phycobilin pigments were determined with different species of algae. As has been generally thought, phycoerythrin forms the major phycobilin pigment in Rhodophyceae, but contrary to the widespread notion, the same pigment was found to be also predominant in most of the blue-green algae investigated.

J. Biochem., **46**, 1259–1261 (1959)

Effect of Pre-Illumination on the Formation of Phycobilin Pigments in a Blue-Green Alga, *Tolypothrix tenuis*

By Akihiko HATTORI and Yoshihiko FUJITA

Division of Biosynthesis II

Effect of pre-illumination on the formation of phycobilin pigments was investigated with a blue-green alga, *Tolypothrix tenuis*.

When the cells pre-illuminated with fluorescent light was incubated with nitrate in the dark, a marked formation of phycoerythrin was observed. On the contrary, in the cells pre-illuminated with red light, phycocyanin was produced. The presence of nitrate was found to be an indispensable condition for the both pigment formations. No formation occurred under anaerobic conditions.

From these results, it was presumed that the photochemical formation of phycobilin pigments are the processes involving certain dark reactions preceded by photochemical reactions, *i.e.*, some precursors of the pigments are accumulated in the light and converted in the dark into the pigments.

Journal of Antibiotics, ser. A vol. XI No. 1 (1958)

Blasticidin S, a New Antibiotic

By Setsuo TAKEUCHI, Kosei HIRAYAMA, Kazaburo UEDA, Heiichi SAKAI & Hiroshi YONEHARA

Division of Antibiotics

A new antibiotic inhibiting bacteria and *Piricularia oryzae* was isolated from the culture broth of *Streptomyces griseochromogenes* which had been found to produce antifungal substances, and named blasticidin S. It was adsorbed by carbon or hydrogen type of a cation exchange resin and eluted with acid aqueous acetone. The further purification by alumina chromatography gave crystalline hydrochloride of blasticidin S. The free base was also crystallized. Blasticidin S free base is soluble in water and acetic acid, but insoluble in other organic solvents. The analytical results of the free base and the pictrate gave the formula of $C_{14}H_{20}O_5N_6$ for this antibiotic. The solution of N/10 HCl had a maximum absorption at 275 mμ.

The antibiotic was toxic to mice by the intravenous injection. It inhibited widely bacteria at concentrations of about 50–100 mcg/ml, but *Pseudomonas* and *Piricularia oryzae* at a concentration of about 5 mcg/ml.

The Journal of Antibiotics 55–58, XII (1959)

Structure of Griseolutein A

By Shoshiro NAKAMURA, E. Lin WANG, Masao MURASE, Kenji MAEDA and Hamao UMEZAWA

Division of Antibiotics

Monomethyl ester of griseolutein A and monomethyl ester of monoacetyl griseolutein A were prepared and the formula $C_{17}H_{14}O_6N_2$ for griseolutein A was established. The reduction of methyl ester of acetyl griseolutein A gave monomethyl ester of griseoluteic acid, which had been obtained from griseolutein B, and 1-methoxy-

4, 9-dihydroxymethylphenazine. Alkaline hydrolysis of griseolutein A gave glycolic acid and griseoluteic acid (1-methoxy-4-hydroxymethyl-9-carboxyphenazine).

Thus, the structure 1-methoxy-4- (hydroxyacetoxy) methyl-9-carboxyphenazine was determined for griseolutein A.

Chemical and Pharmaceutical Bulletin
539–543, **6**, (1958)

Studies on Structure of Griseolutein B, a Streptomyces Antibiotic. 1. Characterization and Degradation

By Shoshiro Nakamura
Division of Antibiotics

Griseolutein-B and -A were purified by the counter-current distribution method and their properties were examined. Diacetylgriseolutein-B was easily purified and crystallized. It had one methoxyl group, two acetyl groups, and one carboxyl group, but no C-methyl group or N-oxide group. Its methyl ester was obtained. Alkaline hydrolysis of diacetylgriseolutein-B gave griseoluteic acid which was crystallized as its monomethyl ester or as monomethyl ester of monoacetylgriseoluteic acid. Distillation of griseoluteic acid with zinc dust gave phenazine, 1-methoxyphenazine, and another compound. This compound was identified as 1-methoxy-4-methylphenazine by comparison with the synthesized sample. 1-methoxy-4-methyl-phenazine was synthesized from aniline and 3-nitro-4-methoxytoluene and its two crystal forms were observed.

Chemical and Pharmaceutical Bulletin
543–547, **6**, (1958)

Studies on the Structure of Griseolutein-B, a Streptomyces Antibiotic. II. Decarboxylation and Periodic Acid Oxidation

By Shoshiro Nakamura
Division of Anibiotics

Decarboxylation of diacetylgriseolutein-B in qinoline with copper gave 1-methoxy-4-hydroxymethylphenazine. The periodic acid oxidation of griseolutein-B gave griseoluteic acid, formic acid, and formaldehyde. From these results a partial structure of griseolutein-B was presented. The reduction of 1-acetoxy-4-methoxycarbonylphenazine with lithium aluminum hydride gave 1-hydroxy-4-hydroxymethylphenazine and 1-hydroxy-4-methylphenazine.

Chemical and Pharmaceutical Bulletin
547–550, **6**, (1958)

Studies on Structure of Griseolutein-B, a Streptomyces Antibiotic. III. The Complete Structure

By Shoshiro Nakamura
Division of Antibiotics

A drastic reduction of diacetylgriseolutein-B monomethyl ester gave 1-methoxy-4, 9-dimethylphenazine. Griseoluteic acid was assumed to be 1-methoxy-4-hydroxymethyl-9-carboxyphenazine. From these results and the periodate oxidation, griseolutein-B was concluded to be 1-methoxy-4-(1,2-dihydroxyethoxy) methyl-9-carboxyphenazine.

The syntheses of 1-methoxy-4, 6-dimethylphenazine and 1-methoxy-4, 9-dimethylphenazine are described.

Kagaku, **29**, 146, (1959)

Equivalent Osmotic Pressure of Microbial Cells

S. Koga, C. Kuribayashi and K. Nunomura

The osmotic pressure of yeast cells (*Saccharomyces cerevisiae*) in suspension was estimated by rheological measurement. The concept of "equivalent osmotic pressure" was proposed and discussed in connection with the so-called active transport of water.

J. App. Physics, Japan, **28**, 173, (1959)

Propagation of Ultrasonic Waves in Aqueous Suspension of Yeast Cell

K. FUKUTOMI, Y. WADA and S. KOGA

The dynamic compressibility and volume viscosity of alive yeast (*Saccharomyces cerevisiae*) in aqueous suspension was estimated through the measurement of the velocity and attenuation of ultrasonic waves in the whole suspension.

J. App. Physics, Japan, **28**, 247, (1959)

A Recording Titrator for Microbiological Uses

S. KOGA, I. KAGAMI and K. NUNOMURA

A simple design for self-recording pH titrator was presented for microbiological process analysis. Titrant was dropwise delivered. The volume of each drop was maintained constant and the numbers of the drops delivered were integrated on a recording instrument.

Genetics, 43, 705 (1958)

Complementary Genes Controlling Homothallism in Saccharomyces

By Toshiaki TAKAHASHI

Division of Microbial Genetics

From the cross between a Carbondale heterothallic strain and a haploid segregant which had arisen from a hybrid between a homothallic and a heterothallic strain of Saccharomyces, zygotes giving the segregation ratio one diploid to three haploid were frequently recovered. To explain this irregular segregation, a set of complementary genes (HM_1 and HM_2) was postulated for the expression of the homothallic character (obligatory diploidization, positive sporulation, and absence of mating reaction). This hypothesis was evidenced by the cross tests between the progenies of the above mentioned zygotes and the Carbondale haploid strain. Due to the results it was concluded that the gene HM_1 did come from the Carbondale strains and the gene HM_2 from the homothallic strains.

Can. J. Microbiol., 4, 571 (1958)

Heterocaryosis and Genetic Recombination in *Streptomyces griseoflavus*

By Hiuga SAITO

Division of Microbial Genetics

Upon prolonged incubation of two auxotrophic mutants from *Streptomyces griseoflavus* 142 plated together on minimal medium, heterocaryotic colonies producing only parental type conidia were formed. In addition, from the mixed culture of two double auxotrophs on a medium supplemented with limited amounts of nutrients, stable recombinant-type conidia were recovered at relatively high frequencies. According to these observations, it is suggested that genetic recombination occurs in heterocaryotic mycelial growth antecedent to conidiation. However, complete synapsis appears to be doubtful because of the unbalanced patterns of recombinant recovery.

Cytologia, 23, 496 (1959)

The Life-cycle of *Streptomyces griseoflavus*

By Hiuga SAITO and Yonosuke IKEDA

Division of Microbial Genetics

On the basis of cytological examination, the life-cycle of *Streptomyces griseoflavus* appears to consist of the following phases: 1) a vegetative phase (a primary mycelium) which develops after conidial germination; and 2) a sporulation phase (a secondary mycelium). Frequently the sporulation phase is the consequence of direct matureation of primary mycelium. The primary

mycelium may give rise to three diverse and distinctive structures; namely, "nests", "swollen bodies" and "clubs". These appear to correspond to "initial cells" discussed in the literature and may represent a transition period between the vegetative and sporulation phases of growth. However, the present investigation suggests that such a transition period is not obligatory in the life-cycle of *Streptomyces griseoflavus*. The secondary mycelium gives rise to conidia which contain single diploid or bipartite nuclei by a process of fragmentation or segmentation.

Zeitschrift für Vererbungslehre, 90, 66 (1959)

Genetic Analysis of α-Methyl-Glucoside Fermentation in Saccharomyces

By Toshiaki Takahashi and Yonosuke Ikeda

Division of Microbial Genetics

The hybrid produced between a Carbondale haploid (α-methyl-glucoside rapid fermenter) and a haploid strain (non- fermenter), derived from a hybrid between a homothallic and a heterothallic Saccharomyces, showed an irregular segregation pattern with regard to the fermentation of this sugar.

To explain this irregularity, three pairs of alleles, MG_1/mg_1, MG_2/mg_2 and MG_3/mg_3, were assumed to be in quantitative control of the fermentation. Haploid cultures carrying the genotypes (1) mg_1 mg_2, mg_3, (2) MG_1 mg_2 mg_3, (3) mg_1 MG_2 mg_3, (4) mg_1 mg_2 MG_3, (5) MG_1 MG_2 mg_3, (6) MG_1 mg_2 MG_3, (7) mg_1 MG_2 MG_3, and (8) MG_1 MG_2 MG_3, were actually recovered. Strains equipped with: either (1) or (2); either (4) or (6); (3); (5); (7); or (8) are non-fermenters, extremely-slow-fermenters, slow-fermenters, medium-fermenters, semi-rapid-fermenters and rapid-fermenters respectively.

The role of these genes in sugar fermentation and the identity or nonidentity of some of these genes with maltose and sucrose genes was discussed.

VOLUME 5 1959

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CONTENTS

Volume 5

Numbers 1–2, March 1959

K. TSUDA, T. ASAI, Y. SATO, T. TANAKA und M. KATO: Mikrobiologische Hydroxylierung der Steroide V (1). Hydroxylierung von Progesteron und Reichsteins Substanz S durch *Diplodia tubericola* (Ell & Ev. Ganb)..... 1

K. TSUDA, H. IIZUKA, E. OHKI, Y. SATO, A. NAITO und M. HATTORI: Mikrobiologische Hydroxylierung der Steroide VI (1). Hydroxylierung von Progesteron und Reichsteins Substanz S durch *Achromobacter kashiwasakiensis* nov. sp. 7

K. KARASAWA, N. TANAKA, H. YONEHARA and H. UMEZAWA: Taxonomical Studies of the Streptomyces Producing Antimycin A-blastmycin Group Antibiotics 13

A. WATANABE: Distribution of Nitrogen-Fixing Blue-Green Algae in Various Areas of South and East Asia 21

T. HASEGAWA: The Lactose Assimilating Species in the Genus *Rhodotorula* 30

S. KOGA, C. KURIBAYASHI and K. NUNOMURA: Studies on the Rheological Volume of Cell Suspensions 35

E. HASE, S. MIHARA and H. OTSUKA: An Additional Note on the Nature of Sulfer-Containing Peptide-Nucleotide Complex Obtained from *Chlorella* and Yeast 43

A. WATANABE, A. HATTORI, Y. FUJITA and T. KIYOHARA: Large Scale Culture of a Blue-Green Alga, *Tolypothrix tenuis*, Utilizing Hot Spring and Natural Gas as Heat and Carbon Dioxide Sources..... 51

H. IIZUKA and K. YAMASATO: *Pediococcus soyae* Nov. sp. Main Lactic Acid Bacteria in "Shoyu Moromi" 58

K. KITAHARA, S. FUKUI and M. MISAWA: Preparation of L-Aspartic Acid by Bacterial Aspartase (Preliminary Report)..... 74

K. KITAHARA and I. KUSAKA: Pleomorphism in Bacterial Cells 1. Filament Formation in *Lactobacillus delbrueckii* and its Relation to Vitamin B_{12} 78

A. WATANABE: On the Mass-Culturing of a Nitrogen-Fixing Blue-Green Alga, *Tolypothrix tenuis*..... 85

Abstract..... 92

Number 3, October 1959

A. NAKAGAWA and K. KITAHARA: "Taxonomic Studies on the Genus *Pediococcus*" 95

T. ASAI, K. AIDA, T. TANAKA, E. OHKI, T. MATSUHISA, Y. TAKEDA and T. INUI: Microbiological Hydroxylation of Steroid IX. Hydroxylation of Steroid by *Syncephalastrum racemosum* (2) 127

Z. SUGISAKI: Studies on L-Valine Fermentation Part 1. Production of L-Valine by *Aerobacter* Bacteria 138

T. ASAI, K. AIDA and K. OISHI: On the Enzymatic Preparation of L-Phenyl-

alanine (Preliminary Report) 150
A. WATANABE: Some Devices for Preserving Blue-Green Algae in Viable State.. 153

Number 4, January 1960

S. UDAKA and S. KINOSHITA: The Fermentative Production of L-Valine by Bacteria 159
A. WATANABE and T. KIYOHARA: Decomposition of Blue-Green Algae as Effected by the Action of Soil Bacteria 175
R. NOMI: Studies on the Classification of Streptomycetes. Part XII. The Microscopic Morphology and Other Characteristics of *Streptomyces hygroscopicus* and Morphologically Related Strains 180
R. NOMI: Studies on the Classification of Streptomycetes. Part XIII. The Microscopic Morphology and Other Characteristics of Whorl Forming Strains.. 191
T. HASEGAWA, I. BANNO and S. YAMAUCHI: A Taxonomic Study on the Genus *Rhodotorula* 1. The Subgenus *Rubrotorula* nov. Subgen 200
S. AIBA, T. YAMADA and K. KOBAYASHI: "An Analysis on Bubbling of Bacteria-borne Air" 213
E. HASE and S. MIHARA: Further Notes on the Nature of Sulfur-Containing Peptide-Nucleotide Complex Obtained from Yeast 221
Abstract 226

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CONTENTS

S. Udaka and S. Kinoshita: The Fermentative Production of L-Valine by Bacteria 159
A. Watanabe and T. Kiyohara: Decomposition of Blue-Green Algae as Effected by the Action of Soil Bacteria 175
R. Nomi: Studies on the Classification of Streptomycetes. Part XII. The Microscopic Morphology and Other Characteristics of *Streptomyces hygroscopicus* and Morphologically Related Strains 180
R. Nomi: Studies on the Classification of Streptomycetes. Part XIII. The Microscopic Morphology and Other Characteristics of Whorl Forming Strains 191
T. Hasegawa, I. Banno and S. Yamauchi: A Taxonomic Study on the Genus *Rhodotorula* 1. The Subgenus *Rubrotorula* nov. Subgen 200
S. Aiba, T. Yamada and K. Kobayashi: "An Analysis on Bubbling of Bacteria-borne Air" 213
E. Hase and S. Mihara: Further Notes on the Nature of Sulfur-Containing Peptide-Nucleotide Complex Obtained from Yeast 221
Abstract 226